

Mutational analysis of the two zinc-binding sites of the *Bacillus cereus* 569/H/9 metallo- β -lactamase

Dominique DE SENY*, Christelle PROSPERI-MEYS*, Carine BEBRONE*, Gian Maria ROSSOLINI**, Michael I. PAGE‡, Philippe NOEL*, Jean-Marie FRÈRE* and Moreno GALLENi*

*Centre d'Ingénierie des Protéines, Institut de Chimie B6, Université de Liège, Sart-Tilman, B-4000 Liège, Belgium,

**Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, I-53100 Siena, Italy,

‡Department of Chemical and Biological Sciences, The University of Huddersfield, Huddersfield, Yorkshire HD1 3DH, U.K.

Abstract

The metallo- β -lactamase BcII from *Bacillus cereus* 569/H/9 possesses a binuclear zinc centre. The mono-zinc form of the enzyme displays an appreciably high activity, although full efficiency is observed for the di-zinc enzyme. In an attempt to assign the involvement of the different zinc ligands in the catalytic properties of BcII, individual substitutions of selected amino acids were generated. With the exception of His¹¹⁶ \rightarrow Ser (H116S), C221A and C221S, the mono- and di-zinc forms of all the other mutants were poorly active. The activity of H116S decreases by a factor of 10 when compared with the wild type. The catalytic efficiency of C221A and C221S was zinc-dependent. The mono-zinc forms of these mutants exhibited a low activity, whereas the catalytic efficiency of their respective di-zinc forms was comparable with that of the wild type. Surprisingly, the zinc contents of the mutants and the wild-type BcII were similar. These data suggest that the affinity of the β -lactamase for the metal was not affected by the substitution of the ligand. The pH-dependence of the H116S catalytic efficiency indicates that the zinc ions participate in the hydrolysis of the β -lactam ring by acting as a Lewis acid. The zinc ions activate the catalytic water molecule, but also polarize the carbonyl bond of the β -lactam ring and stabilize the development of a negative charge on the carbonyl oxygen of the tetrahedral reaction intermediate. Our studies also demonstrate that Asn²⁵³ is not directly involved in the interaction with the substrates.

Keywords: bacterial resistance, dinuclear zinc enzymes, β -lactamase family.

Abbreviations used: AAS, atomic absorption; Capso, 3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid; dNTPs, deoxynucleoside triphosphates; H116S, a mutant bearing a substitution of His¹¹⁶ with serine, etc.; IMP-1, imipenemase-1 ; IPTG, isopropyl β -D-thiogalactoside; LB, Luria-Bertani.

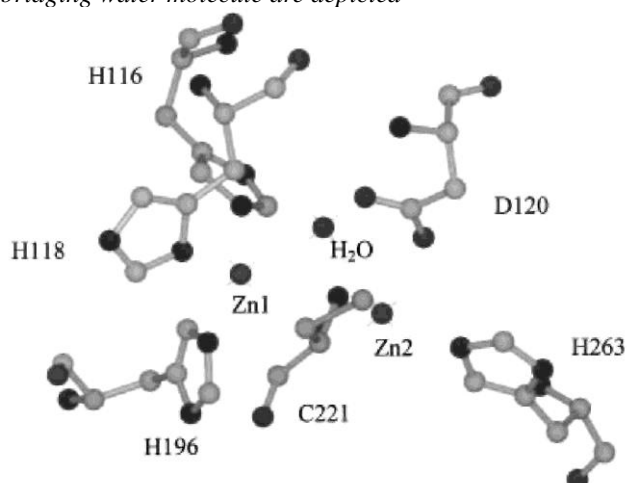
INTRODUCTION

Metallo- β -lactamases are bacterial enzymes that hydrolyse, and thus inactivate, most of the known β -lactam antibiotics. They are included in the molecular class B of the structural classification of β -lactamases and, on the basis of their amino acid sequences, three different lineages, identified as subclasses B1, B2 and B3, have been characterized [1].

The BcII enzyme from *Bacillus cereus* 569H [2], the CcrA enzyme from *Bacteroides fragilis* [3,4] and the imipenemase-1 (IMP-1) enzyme from various Gram-negative pathogens [5-8], which belong to subclass B1, have been studied extensively. Their three-dimensional structures have revealed the presence of two zinc ions in the active site. The first zinc ion (Zn1) is tetrahedrally co-ordinated by three conserved histidine residues (His¹¹⁶, His¹¹⁸ and His¹⁹⁶; the numbering of residues is based on the new BBL numbering scheme proposed for the class B β -lactamases [1]), and by a bridging water/hydroxide ion. The second zinc ion (Zn2) is trigonal bipyramidally co-ordinated by the conserved residues Asp¹²⁰, Cys²²¹ and His²⁶³, the bridging water and an additional water molecule [9-11] (Figure 1). In the case of the *B. cereus* enzyme, the X-ray structures of the mono-zinc [12] and di-zinc forms [9] have been solved. In the mono-zinc form, crystallized at pH 5.7, the metal ion is chelated by the three conserved histidines (His¹¹⁶, His¹¹⁸ and His¹⁹⁶) and the catalytic water molecule. However, perturbed angular correlation (PAC) and EXAFS spectra and kinetic analyses have suggested that, in the mono-Zn form and at neutral pH, the metal could be shared between the two sites [13].

The mono-zinc forms of the BcII and CcrA enzymes exhibit a rather high activity, although full efficiency is only observed for the di-zinc form [14,15]. For BcII, kinetic studies have suggested that the rate-limiting step for the cleavage of the β -lactam ring is controlled by the protonation of the leaving β -lactam nitrogen [16-18]. In contrast, the C-N bond cleavage of nitrocefm by CcrA is thought to occur by N-protonation, and the mechanism apparently involves the formation of an intermediate in which the β -lactam nitrogen has been expelled as the amine anion [19]. In the case of BcII, this intermediate has not been observed. However, Waley and colleagues [16,17] have demonstrated that the hydrolysis of benzylpenicillin proceeds through a branched kinetic pathway containing two non-covalent intermediates, in which the metal was pentaco-ordinated and tetraco-ordinated respectively. Recently, Bounaga et al. [18] revisited the mechanism of the mono-zinc species of BcII. The $k_{\text{cat}}/K_{\text{m}}$ values for benzylpenicillin and cephaloridine hydrolysis showed an inverse second-order-dependence towards pH, indicating that hydrolysis was suppressed by two protonation events. A pK_{a} value of 5.6 was assigned both to the Zn(II)-bound water and to Asp¹²⁰.

Figure 1 Representation of the atomic model of the active site of BcII. Amino acids that ligate with zinc atoms and the bridging water molecule are depicted



Additional studies on benzylpenicillin hydrolysis in the presence of methanol failed to demonstrate the presence of an anhydride intermediate, leaving the Zn(II)-bound hydroxide as the only possible nucleophile. It has also been demonstrated that the carbonyl group of the β -lactam moiety was in direct interaction with the metal ion [20]. At present, the most favoured mechanism involves nucleophilic attack on the β -lactam carbonyl moiety by the Zn(II)-bound hydroxide, and the subsequent formation of a formally monoanionic tetrahedral intermediate. This non-covalent complex is stabilized by interactions with Zn(II) and Asn²³³. Deprotonation by Asp¹²⁰ leads to the formation of a dianionic intermediate. Breakdown of this highly unstable species by C-N bond fission occurs in concert with the protonation of the nitrogen by Asp¹²⁰, now acting as a general acid catalyst. In addition, Paul-Soto et al. [13] have also suggested that, in mono-zinc BcII, Zn(II) acts as a Lewis acid catalysis by polarizing the carbonyl group and makes the metal-bound water nucleophilic, which is oriented further by Asp¹²⁰. Substitution of the unique active-site cysteine residue almost completely abolished the activity of the mono-zinc form, but only reduced the catalytic efficiency of the enzyme when both zinc-binding sites were fully saturated. These data demonstrate the importance of this residue in the catalytic pathway of the mono-zinc enzyme. Previous studies performed on the metallo- β -lactamase produced by *B. cereus* 5/B/6 revealed that residues Glu⁶⁴ and Glu²⁶⁵ were not involved in the catalytic mechanism, but that the His¹¹⁸ zinc ligand was crucial for the β -lactamase activity [21-23]. Substitution of His¹¹⁸ with a serine led to a completely inactive enzyme. Finally, the same authors reported that the H55N mutation modified the substrate profile of the enzyme [23]. The activity of the mutant towards cephalosporin C was decreased, but that against ampicillin was similar to that observed for the wild-type enzyme.

On the basis of both the first BcII structure showing one zinc ion in the 3-His binding site and the high activity of the mono-zinc enzyme, the histidine site is traditionally considered as the catalytic site. The resolution of the di-zinc enzyme structure has underlined the importance of the second binding site, but its exact role in catalysis has not been well established. However, the properties of the Cys²²¹ mutants suggest an important involvement of the 'second' binding site, even in the mononuclear species.

In order to analyse the relative catalytic importance of both binding sites, we obtained a set of BcII derivatives harbouring selected amino acid substitutions of the zinc ligands and nearby residues. The implication of these residues in metal binding and enzymic activity was studied, and the effects of the mutations on the protein stability were analysed.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli DH5 α and BL21(DE3) strains were used for genetic constructions and protein overproduction respectively. The pET9a plasmid was obtained from Merck, Eurolab (Leuven, Belgium). The pBCSK and the pCRTMII were purchased from Stratagene (La Jolla, CA, U.S.A.) and Invitrogen (San Diego, CA, U.S.A.), respectively.

Chemicals

Primers for mutagenesis were obtained from Amersham Bio-sciences (Uppsala, Sweden). Deoxynucleoside triphosphates (dNTPs), Goldstar polymerase and restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), Eurogentec (Liège, Belgium), Biolabs (Beverly, MA, U.S.A.) and Appligene (Illkirch, France). The Quick ChangeTM site-directed mutagenesis kit was from Stratagene; isopropyl β -D-thiogalactoside (IPTG) was

from Eurogentec, chloramphenicol and kanamycin were from Sigma (St. Louis, MO, U.S.A.), benzylpenicillin was from Rhône-Poulenc (Paris, France), nitrocefm was from OXOID® (Basingstoke, Hants, U.K.), cefotaxime was from Roussel UCLAF (Paris, France), cephaloridine was from Sigma, and cefoxitin and imipenem were from Merck Sharp Dohme (Brussels, Belgium).

Plasmid constructions

The pET-BcII plasmid encoding the *B. cereus* 569/H/9 Zn²⁺- β -lactamase [13] was used to introduce the *Nde*I restriction site at the beginning of the gene encoding the mature protein by PCR. For this purpose, the first 360 bp of the gene encoding the mature protein were amplified using the following primers: PN1, 5'-TCTGTACATATGTCACAAAAGGTAGAGAAAACAGT-AATAAAA-3'; and PN2, 5'-TTTCTTTGCTAGTTCTGCAG TTAATGCTG-3'. [The underlined residues denote the positions of the restriction sites for PN1 (*Nde*I) and PN2 (*Bam*HI).] The reaction mixture contained 2.5 mM MgSO₄, 1 μ g/ml template DNA, 1 nmol/ μ l of each primer, 1 mM dNTPs and 1 unit of Goldstar polymerase. Reaction conditions were 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min 30 s at 72 °C, and 5 min at 72 °C in a total volume of 100 μ l. The amplified fragment was gel-purified and introduced into the pCRTMII vector to yield plasmid pCIP101. The gene was sequenced completely to verify the insertion of the *Nde*I restriction site and the absence of unwanted mutations. Sequencing was accomplished with the help of an automated laser fluorescent DNA sequencer (Amersham Biosciences). pCIP101 was digested with *Eco*RI and *Pst*I. The 370 bp fragment was gel-purified and ligated into the pBC-BcII plasmid containing a cloned copy of the BcII gene that was digested with the same enzymes, yielding plasmid pCIP102, which contains the coding sequence of the mature protein and was used for site-directed mutagenesis. The expression plasmid (named pCIP103) was obtained by digesting pCIP102 with *Nde*I and *Bam*HI, and inserting the corresponding fragment in pET9a digested with the same enzymes.

Site-directed mutagenesis

The H116S, H118S, H196S, H263S, D120N and N233Y mutants of the *B. cereus* 569/H/9 Zn²⁺- β -lactamase were obtained according to the instructions in the Quick ChangeTM site-directed mutagenesis kit. The mutations were performed in pCIP102, and the mutagenic primers (forward and reverse) are shown in Table 1. Reaction conditions used were: 5 min at 95 °C, 16 cycles of 30 s at 95 °C, 1 min at 60 °C and 9 min at 68 °C, and 5 min at 68 °C. After plasmid amplification, *E. coli* DH5 α cells were transformed by the PCR product, and colonies were isolated on Luria-Bertani (LB) agar plates containing 30 μ g/ml chloramphenicol. The desired mutants were identified by DNA sequencing on miniprep columns.

The BcII gene containing the mutation was cloned into the pET9a plasmid. The different mutated genes were isolated from pCIP102 by digestion with the restriction enzymes *Nde*I and *Bam*HI. They were cloned into pET9a digested with the same enzymes to yield pCIP103-H116S, pCIP103-H118S, pCIP103-H196S, pCIP103-H263S, pCIP103-D102N and pCIP103-N233Y. The C221A and C221S mutants were produced and purified as described by Paul-Soto et al. [13].

Enzyme purification

In the different pCIP103 plasmids, the genes encoding the wild-type and mutant Zn²⁺- β -lactamase were under the transcriptional control of the T7 promoter. They were expressed in *E. coli* BL21(DE3) as soluble intracellular proteins. The bacteria were cultured overnight at 37 °C in 500 ml of LB medium containing 50 μ g/ml kanamycin as the selection agent. This initial culture was added to 15 litres of the same medium. At a *D*₆₀₀ of 0.6, 100 μ M IPTG was added, and the culture was grown for a further 3 h. The bacteria were harvested by a 15 min centrifugation at 5000 g and resuspended in 500 ml of 10 mM Hepes, pH 7.5, containing 100 μ M ZnSO₄ (buffer A). The cells were broken with the aid of a cell disintegrator (Serie Z; Constant System, Warwick, U.K.). Cell lysates were centrifuged at 30 000 g for 30 min, and the supernatant was loaded on to an SP Sepharose Fast Flow column (2.6 cm \times 34 cm; Amersham Biosciences) equilibrated in buffer A. The β -lactamase was eluted at a rate of 5 ml/min using a linear salt gradient (0-1 mM NaCl) in 800 ml of buffer A. The active fractions were dialysed overnight against buffer A, and then loaded on to an UnoTM S12 continuous-bed ion-exchange column (15 mm \times 68 mm; Bio-Rad, Brussels, Belgium) equilibrated in buffer A. The β -lactamase was eluted at a rate of 2 ml/min using a linear salt gradient (0-0.6 M) of 240 ml in buffer A. The active fractions were concentrated to 1 mg/ml, as determined by measuring absorbance at 280 nm and using the published ϵ of 30500 M⁻¹ \cdot cm⁻¹ [13]. The protein sample was finally dialysed against buffer A containing 0.2 M NaCl. The purity and the molecular masses of all purified proteins were confirmed by determining the *M*_r values with the aid of an electrospray mass spectrometer (VG Bio-Q) upgraded with a Platform source (Micromass, Altrincham, Cheshire, U.K.). The samples (100 pmol) were dissolved in formic acid/acetonitrile (1 : 1000, v/v) in water, and injected into the source of the mass spectrometer with a syringe pump (Harvard Instruments, South Natick, MA, U.S.A.) at a flow rate of 6 μ l/min.

Metal content analysis

The zinc content of the enzyme was measured by atomic absorption (AAS) in the flame mode with an Perkin-Elmer 2100 spectrometer. Before the metal analyses, 1 ml of protein samples (40 μ M) was dialysed three times over a period of 8 h against 1 litre of doubly distilled metal-free water containing 10 mM Hepes/0.2 M NaCl, pH 7.5, at 4 °C. The

zinc content of this dialysis buffer was approx. 20 nM. The assays were performed in either the absence or presence of 100 μ M ZnSO₄ in the dialysis buffer, and the samples dialysed against the buffer containing 100 μ M zinc were concentrated by Centricon ultrafiltration to attain a final protein concentration in excess of 40 μ M. The flowthrough was used as a blank, and the reference for the samples without added zinc was the final dialysis buffer. The protein concentration was determined spectrophotometrically by using $\epsilon_{280} = 30500 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Metal content values reported for each sample are averages of results obtained from three independent experiments. The buffers used for the determination of the pH-dependence of zinc content were 10 mM acetate (pH 5), Mes (pH 6), Hepes (pH 7.0, 7.5 and 8) and Taps (pH 9). At each pH value, the experiments were performed as described above.

Kinetic studies

The hydrolysis of all antibiotics was monitored by following the absorbance variation 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 path lengths were used, depending on the substrate concentration. The absorption coefficient variations used ($\Delta\epsilon$) were as follows : benzylpenicillin, $\Delta\epsilon_{235} = -775 \text{ M}^{-1} \cdot \text{cm}^{-1}$; nitrocefin, $\Delta\epsilon_{482} = 17500 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cefotaxime, $\Delta\epsilon_{260} = -7500 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cephaloridine, $\Delta\epsilon_{260} = -10000 \text{ M}^{-1} \cdot \text{cm}^{-1}$; ceftiofex, $\Delta\epsilon_{260} = -6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$; and imipenem, $\Delta\epsilon_{300} = -9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The kinetic parameters were determined either from the initial rates of reactions, using both Hanes' linearization of the Henri-Michaelis equation and a direct nonlinear regression with the hyperbolic equation, or by analysing the complete hydrolysis time courses, as described by De Meester et al. [24]. The reported $k_{\text{cat}}/K_{\text{m}}$ values are the means for at least three experiments, in which the different enzymes were added to the substrate solutions prepared in buffers containing the stated Zn²⁺ concentrations. All experiments were performed at 30 °C in 10 mM Hepes, pH 7.5/0.2 M NaCl.

pH-dependence of enzyme activity

The buffers used (10 mM) were sodium acetate (pH 4-5), Mes (pH 6), Mops (pH 7-8), Taps (pH 9) and Capsos (3-[cyclohexyl-amino]-2-hydroxy-1-propanesulphonic acid; pH 10) at 30 °C, with the ionic strength increased to 0.2 M with KCl. The H196S mutant concentration ranged from 2.3-11.5 μ M, and the benzylpenicillin concentration was 0.5 mM. Hydrolysis of benzylpenicillin was monitored by measuring the decrease in absorbance at 235 nm. The kinetic parameters were determined as described previously, and curve fitting was achieved using ENZFITTER (Elsevier Biosoft, Cambridge, U.K.) Repeat hydrolysis reactions, brought about by injecting a second sample of substrate into the reaction mixture after completion of hydrolysis of the first aliquot, gave identical pseudo-first-order rate constants. The experiments were performed with 1, 5, 100 and 1000 μ M ZnSO₄ in the buffer for the H196S mutant. The equation used for the best curve fitting was as follows :

$$k_{\text{cat}}/K_{\text{m}} = \frac{(k_{\text{cat}}/K_{\text{m}})_{\text{max}}}{1 + [\text{H}^+]/K_{\text{a1}} + [\text{H}^+]^2/K_{\text{a1}}K_{\text{a2}} + K_{\text{a3}}/[\text{H}^+]}$$

CD measurements

Conservation of secondary and tertiary structures was verified by CD spectroscopy. The purified enzymes were diluted to a final concentration of 0.4 mg/ml in 10 mM Hepes, pH 7.5/0.2 M NaCl. A Jobin Yvon CD6 spectropolarimeter was used to collect spectra at 25 °C in the far- (190-250 nm) and near- (250-360 nm) UV regions. CD spectra in the near- and far-UV regions of the wild-type and H196S enzymes were also collected at different pH values (pH 4.5 with buffer 10 mM sodium acetate; pH 7.5 with buffer 10 mM Mops; and pH 9 with buffer 10 mM Capsos).

RESULTS

Overexpression and purification of wild-type BcII and BcII mutants

After production of the different enzymes in *E. coli* BL21(DE3) and their purification to homogeneity, a yield of 15-40 mg of β -lactamase per litre of culture was typically obtained, but only 5 mg/l was obtained for H116S and H118S. Surprisingly, a slight pink colouration was present in the purified wild-type BcII. Determination of the molecular mass of the enzyme indicated the presence of a 48 Da increase compared with the calculated mass. However, extensive dialysis against buffer A resulted in the disappearance of this colouration. At this stage, the N-terminal amino acid sequence and the measured molecular mass were as expected. The colouration was not observed for the mutants; furthermore, their measured molecular masses were in good agreement with the calculated ones. The near- and far-UV CD spectra of all the mutants were superimposable on that of the wild-type BcII, showing that the point mutations did not cause a large conformational change in the structure of the enzyme (results not shown).

Table 1 List of mutagenic primers (5' - 3') used to generate mutants. The modified bases are underlined. For, forward primer; rev, reverse primer.

Primers	Sequences
H116S _{for}	CGTAACGGATGTCATTATTACAT <u>CT</u> GCGCACGCTGATCGAATTGGC
H116S _{rev}	GCCAATTCGATCAGCGTGCGCAG <u>AT</u> TGTAATAATGACATCCGTTACG
H118S _{for}	GGATGTCATTATTACACATGCGT <u>CC</u> GCTGATCGAATTGGCGGAATA
H118S _{rev}	TATTCCGCCAATTCGATCAGCGG <u>AG</u> CGCATGTGTAATAATGACATCC
H196S _{for}	AACATTTTATCCAGGGAAAGGGT <u>CT</u> TACAGAAGATAATATTGTCGTA
H196S _{rev}	TACGACAATATTATCTTCTGTAG <u>AC</u> CCCTTTCCCTGGATAAAATGTT
H263S _{for}	TATAAATGCAGTAGTGCCTGGT <u>CT</u> TGGGGAAGTAGGGGACAAAGGA
H263S _{rev}	TCCTTTGTCCCCTACTTCCCCAG <u>AA</u> ACCAGGCACTACTGCATTTATA
D120N _{for}	ATTATTACACATGCGCACGCTT <u>TAC</u> GAAATTGGCGGAATAAAAACG
D120N _{rev}	CGTTTTTATTCCGCCAATTCGT <u>AAA</u> AGCGTGCGCATGTGTAATAAT
N233Y _{for}	TACGTCCGCGAAAGATTAGGAT <u>AT</u> GTTGCGGATGCTTATGTAAATG
N233Y _{rev}	CATTTACATAAGCATCCGCAAC <u>ATAT</u> TCCTAAATCTTTCGCGGACGTA

Table 2 Zinc ion content determined by AAS for the BcII enzyme and the mutants with or without additions of 100 μ M zinc sulphate in 10 mM Hepes containing 0.2 M NaCl at pH 7.5

	[Zn]/[E]	
Enzyme	[Zn ²⁺] = 0.4 μ M	[Zn ²⁺] = 100 μ M
Wild-type	1.4 \pm 0.1	1.7 \pm 0.1
D120N	1.4 \pm 0.1	1.6 \pm 0.1
C221S	1.2 \pm 0.1	1.7 \pm 0.1
H116S	1.0 \pm 0.05	2.0 \pm 0.1
H118S	0.95 \pm 0.05	1.7 \pm 0.1
H196S	1.0 \pm 0.2	1.7 \pm 0.1
H263S	1.0 \pm 0.1	1.1 \pm 0.2

Metal content of BcII mutants at pH 7.5

The zinc-ion content of the wild-type enzyme and the mutants is summarized in Table 2. With no zinc added to the buffer ([Zn²⁺] = 0.4 μ M), the wild-type enzyme contained 1.4 \pm 0.1 mol of zinc/mol of protein. The D120N and C221S mutants exhibited similar zinc contents, whereas that of the other mutants was slightly lower, in the range 0.9-1.2 mol/mol of protein. After addition of 100 μ M zinc to the dialysis buffer, the metal content of the BcII enzyme increased to 1.7 \pm 0.1 mol. Under the same conditions, all the mutants were shown to bind between 1.6-2.0 mol of zinc, with the exception of the H263S mutant, which did not bind more than one equivalent of zinc.

Kinetic studies

The catalytic efficiency of the BcII mutants against a set of β -lactam antibiotics was compared with that of the wild-type enzyme at pH 7.5, at both low (0.4 μ M) and high (100 μ M) Zn²⁺ concentrations. Substitution of the residues of both zinc-binding sites resulted in a significant decrease in enzymic activity (Tables 3 and 4). The activities of H118S, H196S, H263S and D120N are affected the most by the mutations, whereas H116S and N233Y are less affected by the mutagenesis. The decrease in catalytic efficiency of H118S, H196S and H263S against benzylpenicillin and nitrocefm was mainly due to an increase in K_m (Table 3), although the k_{cat} value was also affected. H116S was the only mutant having a k_{cat} value similar to that of the wild-type enzyme.

The kinetic studies started by Paul-Soto et al. [13] on the C221A mutant with benzylpenicillin and nitrocefm were augmented by studies with imipenem, cephaloridine and cefotaxime (Table 4). The same increase in activity was observed with all of these substrates in the presence of 100 μ M zinc relative to 0.4 μ M, a finding which can be correlated with the zinc content of the protein. The di-zinc form of the C221A mutant exhibits an appreciably high activity, whereas its mono-zinc form is almost inactive. The same behaviour was observed with the C221S mutant. The increase in catalytic efficiency measured with benzylpenicillin and nitrocefm in the presence of zinc is mainly due to an increased k_{cat} value. A catalytic efficiency of 200 M⁻¹ · s⁻¹ against cefoxitin was also measured for the wild-type enzyme at a zinc concentration of 100 μ M. As the hydrolysis of this substrate occurred too slowly to be directly monitored with the

H196S and H263S mutants, an inhibition constant was determined in the presence of nitrocefm or imipenem as a reporter substrate (results not shown). The measured K_i values were 90, 190 and 90 μM for the wild-type, H196S and H263S enzymes respectively. These values increased 10- to 20-fold in the presence of 100 μM zinc.

Table 3 Kinetic parameters with respect to benzylpenicillin and nitrocefin for the wild-type (WT) enzyme and the mutants in 10 mM Hepes/0.2 M NaCl at pH 7.5 and 30 °C. The zinc concentration was approx. 0.4 μM . Standard deviation values were < 5%. ND, not determined.

Enzyme	Benzylpenicillin			Nitrocefin		
	$K_m(\mu\text{M})$	$k_{\text{cat}}(\text{s}^{-1})$	$k_{\text{cat}}/K_m(\text{M}^{-1} \cdot \text{s}^{-1})$	$K_m(\mu\text{M})$	$k_{\text{cat}}(\text{s}^{-1})$	$k_{\text{cat}}/K_m(\text{M}^{-1} \cdot \text{s}^{-1})$
WT	170	230	1360000	9	43	4900000
H116S	530	47	89000	180	44	227000
H118S	2300	36	12200	220	5	21600
H196S	5700	7	1230	160	0.2	1400
H263S	> 6000	>14	2600	365	1.3	3600
C221S	475	15	31000	8	1.3	166000
C221S (100 μM Zn^{2+})	775	200	262000	21	63	3000000
D120N	440	3.5	7900	133	2.2	16500
N233Y	ND	ND	ND	17	6	330000

Table 4 Catalytic efficiency of the wild-type (WT) enzyme and the mutants with respect to imipenem, cephaloridine and cefotaxime in 10 mM Hepes/0.2 M NaCl and 0.4 μM or 100 μM ZnSO_4 at pH 7.5 and 30 °C. Standard deviations were below 5%. ND, not determined.

Antibiotics	$[\text{Zn}^{2+}](\mu\text{M})$	Enzyme ...	$k_{\text{cat}}/K_m(\text{M}^{-1} \cdot \text{s}^{-1})$							
			WT	H118S	H196S	H263S	C221S	C221A	D120N	N233Y
Imipenem	0.4		170000	5300	1500	2000	1000	400	2500	28000
	100		220000	8800	3000	2400	16000	4000	2800	110000
Cephaloridine	0.4		11500	ND	1500	ND	< 1000	< 1000	ND	ND
	100		46000	ND	1600	190	6600	11000	ND	ND
Cefotaxime	0.4		820000	ND	4000	< 500	1000	650	ND	ND
	100		1300000	ND	14500	2500	130000	200 000	ND	ND

Effect of zinc ion concentration on the kinetic parameters

The zinc-dependence of the catalytic efficiencies of the wild-type enzyme and the H196S, H263S, C221S and C221A mutants was studied with nitrocefin as the substrate (Table 5), but also with imipenem, cefotaxime and cephaloridine (results not shown). Only nitrocefin and benzylpenicillin were used for the H116S and H118S mutants (Table 5). The effect of zinc on the activity of D120N and N233Y was also determined by initial rate measurements with benzylpenicillin (results not shown). In all cases, the catalytic efficiency of the different enzymes was slightly higher in the presence of increased concentrations of zinc. However, this effect was much more important for the cysteine mutants when compared with the wild-type enzyme and the other mutants (Table 5).

pH-dependence of the metal content of the wild-type enzyme and the H196S mutant

The zinc-ion content of the wild-type and the H196S enzymes was measured by AAS from pH 5-9, and the results are summarized in Table 6. At pH 5, with a protein concentration of 20 μM and with approx. 0.4 μM zinc in the buffer solution, the amount of zinc bound to the protein was very low. However, in the presence of 100 μM zinc, the wild-type enzyme contained 1 equivalent of zinc/mol of protein. Under the same conditions, the metal content of the H196S mutant remained at < 1 equivalent of zinc/mol of protein. At pH 6 and in the presence of 100 μM Zn^{2+} , wild-type BcII and the H196S mutant contained 1.4 and approx. 1 equivalent of zinc per molecule respectively. In the range pH 7-9, the zinc-ion content of the wild-type enzyme increased slightly, and was stabilized at a value of 1.7. The binding of the second zinc ion to the H196S mutant became significant at pH 7 in the presence of 100 μM zinc, and reached a value of 1.7 equivalents of zinc/mol of protein at pH 7.5 (Table 6).

Table 5 Zn ion concentration-dependence of the catalytic efficiency of the BcII wild-type (WT) and mutants compared with the zinc-ion concentration. The catalytic efficiencies of the BcII wild-type enzyme, the histidine and the cysteine mutants were determined at different zinc ion concentrations (0.4-100 μM). The substrate was nitrocefin. Standard deviations were in the range 10-20%. ND, not determined.

		$k_{\text{cat}} / K_{\text{m}} (\text{M}^{-1} \cdot \text{s}^{-1})$						
$[\text{Zn}^{2+}] (\mu\text{M})$	Enzyme ...	WT	H116S	H118S	H196S	H263S	C221S	C221A
0.4		1.5×10^6	2.4×10^5	4.2×10^4	6.6×10^3	2.1×10^3	2.5×10^3	10^3
10		2.3×10^6	ND	6×10^4	1.3×10^4	3.2×10^3	3.4×10^5	8.7×10^4
20		ND	3×10^5	5.9×10^4	1.1×10^4	4.3×10^3	3.4×10^5	8.7×10^4
50		ND	ND	ND	ND	ND	3.7×10^5	1.1×10^5
100		3.1×10^6	3.3×10^5	7.6×10^4	1.35×10^4	10^4	6.8×10^5	2.8×10^5

Table 6 pH-dependence of the metal content for the wild-type enzyme and the H196S (40 μM) mutant in 10 mM Hepes containing 0.2 M NaCl and 0.4 or 100 μM ZnSO_4 . Standard deviations were + 0.1 equivalent of Zn/mol of enzyme.

		$[\text{Zn}]/[\text{E}]$			
		Wild-type		H196S	
pH	$[\text{Zn}^{2+}] (\mu\text{M})$...	0.4	100	0.4	100
5		0.35	0.9	0.15	0.15
6		1.3	1.4	0.55	0.85
7		1.4	1.6	0.95	1.5
8		1.5	1.7	1.35	1.7
9		1.7	1.7	1.6	1.7

pH effect on catalytic efficiency

A pH profile of the wild-type BcII catalytic efficiency was obtained by Bounaga et al. [18]. The same study was realized on one of the most kinetically altered mutants, H196S, in order to understand its loss of activity. Figures 2(a) and 2(b) show the $k_{\text{cat}}/K_{\text{m}}$ rate constant and a plot of $\log k_{\text{cat}}/K_{\text{m}}$ against pH respectively for the hydrolysis of benzylpenicillin by wild-type BcII [18] and the H196S mutant in the presence of two distinct zinc ion concentrations (100 μM and 1 mM). Repeated hydrolysis reactions, brought about by injecting a second sample of substrate into the reaction mixture after completion of hydrolysis of the first aliquot, gave identical pseudo-first-order rate constants and demonstrated that no acid-catalysed inactivation or degradation of the protein had occurred. Characteristic 'bell-shaped' curves were obtained for both enzymes, although the loss of activity of the H196S mutant is significant at all pH values when compared with the wild-type enzyme. The mutant appears to display the two acidic pK_{a} values that could not be separated in the wild-type enzyme. There is one inflection at a pH of approx. 5.2, and another at approx. pH 7. These presumably correspond to Asp¹²⁰ for the lower pK_{a} , and the zinc-bound water, the pK_{a} of which has now increased to 7 due to the replacement of the histidine by a serine. The pK_{a} values derived from the best-fit curves are $\text{pK}_{\text{a}1} = 5.3$, $\text{pK}_{\text{a}2} = 6.9$ and $\text{pK}_{\text{a}3} = 9.5$.

The catalytic efficiencies of the H196S mutant were compared in the presence of 100 μM and 1 mM ZnSO_4 between pH values of 4 and 8. The increased metal ion concentration resulted in a shift of the acidic part of the bell-shaped curve to the left, yielding a higher activity against benzylpenicillin at lower pH values; nevertheless, the overall shapes of the curves were similar. At pH 8, the measured $k_{\text{cat}}/K_{\text{m}}$ values were not significantly different at the two zinc concentrations. The effect of zinc on the catalytic efficiency at pH 5 and 7 is summarized in Table 7. The results show that, at pH 5, the mutant activity increases in the presence of the zinc ion. There was no evidence from the CD spectrum for any rapid and reversible denaturation of H196S at pH 4.5 and pH 9 (results not shown). In addition, at these two pH values and in the presence of 1 mM zinc ions, the maximal level of activity of H196S is not attained. Although we did not measure the metal content of the mutant at an external zinc ion concentration of 100 μM , we believe that only partial occupancy of the second metal-binding site was observed.

Figure 2 pH-dependence of k_{cat}/K_m for the H196S mutant. (a) The catalytic efficiency of the H196S was determined at two zinc concentrations $\{[Zn^{2+}] = 100 \mu M (\bullet) \text{ and } 1 \text{ mM} (\circ)\}$ from pH 4-8 and at $100 \mu M$ at pH 9 and 10. Standard deviation values were below 15%. The best-fit curve for the variation of k_{cat}/K_m with pH is shown (—). (b) Comparison between the $\log(k_{cat}/K_m)$ for the wild-type and H196S enzymes $\{\blacktriangle, \text{wild-type } ([Zn^{2+}] = 2-40 \mu M); \triangle, \text{wild-type } ([Zn^{2+}] = 1 \text{ mM}); \circ, \text{H196S } ([Zn^{2+}] = 1 \text{ mM}); \bullet, \text{H196S } ([Zn^{2+}] = 100 \mu M)\}$. The data for the BcII wild-type are from Bounaga et al. [18].

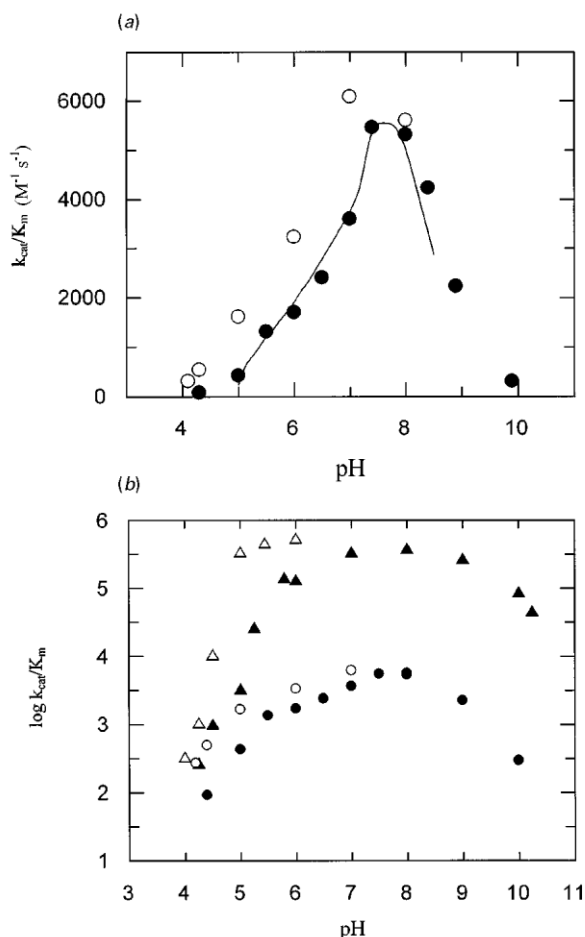


Table 7 Effect of the zinc(II) concentration on the H196S mutant activity against benzylpenicillin at pH 5 and 7. Standard deviation values were < 5%.

pH	$k_{cat} / K_m (M^{-1} \cdot s^{-1})$			
	$[Zn^{2+}] \dots 5 \mu M$	$10 \mu M$	$100 \mu M$	1 mM
5	100	250	430	1700
7	2200	2800	3700	6200

DISCUSSION

Modifying the zinc ligands of the native enzyme could have the following effects: (i) alteration of the strength of metal-ion binding to the protein; (ii) changing the co-ordination number, geometry and effective charge density on Zn; (iii) changing the pK_a of the bound water, which is thought to act as the nucleophile. Any of these could have a profound effect on the catalytic activity of the enzyme, some of which would also be dependent on pH and external zinc-ion concentration. The overall effect depends on the role of Zn in the catalytic mechanism used by the wild-type enzyme. Decreasing the effective positive charge on Zn is expected to: (i) increase the pK_a of the Zn-bound water, thus rendering it a better nucleophile, but decreasing the concentration of the metal-bound hydroxide-ion form at pH values below the pK_a ; (ii) decrease the effectiveness of the zinc-bound water if it acted as a general acid catalyst by, for example, facilitating C-N bond fission; (iii) decrease the ability of Zn to act as a Lewis acid in stabilizing the development of negative charge density on the lactam carbonyl oxygen following nucleophilic attack. The observed effect of zinc ligand modifications that decrease the positive charge density on the metal and increase the pK_a of the bound water will depend on the pH, the mechanism and the relative importance of each factor in bond making and breaking. Using site-directed mutagenesis, seven different β -lactamase mutants with amino acid substitutions in or near

the active site were obtained to address these questions. The histidines were replaced by serines. In order to not perturb drastically the structure of the active site, we decided to replace the histidine residues with another hydrophilic amino acid, which can play the role of a weak metal ligand. The hydrolytic activities and metal ion contents of the different enzymes were analysed, and the results indicated that the majority of the substituted side chains have a role in the enzymic function of BcII.

Analysis of the first binding site

His¹¹⁶, His¹¹⁸ and His¹⁹⁶ are the three histidines constituting the so-called 'first zinc-binding site'.

His¹¹⁶

Substitution of His¹¹⁶ with serine did not drastically affect the catalytic efficiency or the zinc-binding capability of BcII. The overall 20-fold decrease in catalytic efficiency for nitrocefin is due to a large increase in the K_m value, rather than a decrease in k_{cat} , whereas both parameters are affected for benzylpenicillin. H116S binds one or two zinc ions in the presence of zinc concentrations of 0.4 or 100 μ M respectively. The dependence of the k_{cat}/K_m values on zinc-ion concentrations for H116S and the wild-type enzyme was found to be similar. A small increase in the k_{cat}/K_m values was observed in the presence of increased external zinc-ion concentrations. Substitutions of His¹¹⁶ with asparagine and alanine were performed in CcrA [25] and IMP-1 [26], two other members of the subclass B1, respectively. The CcrA mutant was still able to bind two zinc ions without added zinc in the buffer, whereas the IMP-1 mutant was only able to bind one. Unlike the BcII mutant, the activity of IMP-1 H116A increased drastically in the presence of a large excess of zinc to reach a value similar to that obtained for the wild-type enzyme. The decrease in catalytic efficiency of the CcrA and IMP-1 mutants towards cephalosporins was mainly due to increased values of K_m , as observed for BcII. Replacement of His¹¹⁶ with an asparagine in CcrA significantly reduced the k_{cat} value with respect to benzylpenicillin and imipenem. The presence of the asparagine side chain in CcrA might impede a correct positioning of the penicillins and carbapenems in the catalytic site.

His¹¹⁸ and His¹⁹⁶

Alteration of the kinetic parameters of H118S and H196S was more pronounced. For benzylpenicillin and nitrocefm, a decrease in the k_{cat} value, combined with an increase in the K_m value by a factor of 10, resulted in a lowering of the catalytic efficiency of the mutants by 2-3 orders of magnitude. The same situation occurred with imipenem and cefotaxime. Cephaloridine remained the only tested substrate that was hydrolysed appreciably by H196S, for which the overall reduction in k_{cat}/K_m was approx. 20-fold. Our kinetic data on H118S are in good agreement with the preliminary observation with *E. coli* cells containing the H118Y mutant gene. The production of the mutant did not increase the resistance of the *E. coli* cells to β -lactam antibiotics [23].

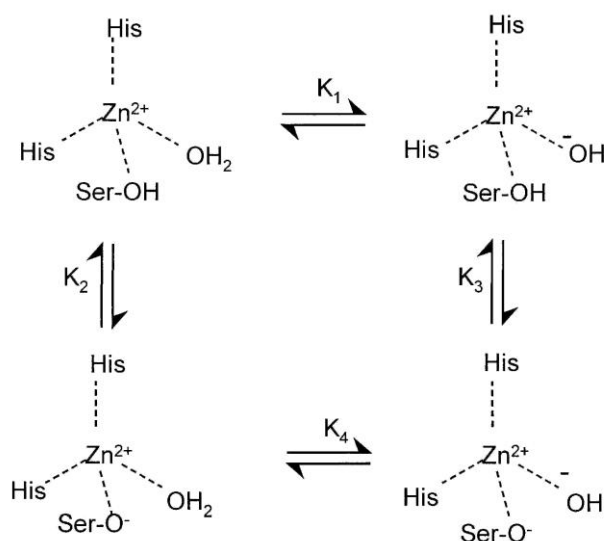
The substitution of H¹¹⁸ and H¹⁹⁶ with alanine in the IMP-1 enzyme yielded two inactive enzymes in the presence of low zinc concentrations [26]. For H116A IMP-1, a final concentration of 1 mM zinc sulphate yielded k_{cat} and K_m values that were higher than those obtained for the wild-type enzyme. However, a different behaviour was observed for the BcII H118S and H196S mutants. Addition of zinc ions did not modify their pattern of activity. Only a slight increase in the catalytic efficiency was noted. At pH 7.5, H118S and H196S were able to bind one and two zinc ions at external metal concentrations of 0.4 and 100 μ M respectively. The study of the pH-dependence of the $[Zn]/[E]$ ratio for the H196S and wild-type enzymes indicated that, below pH 8, the zinc content of H196S was always lower than that of the wild type, even at 100 μ M zinc. As confirmed by X-ray crystallography, at pH 5 and a zinc concentration of 100 μ M, only the mono-zinc BcII wild-type β -lactamase structure can be obtained [9]. These data also show that the affinity constant of the two zinc ions for the binding site is pH-dependent, and this observation is even more relevant for the H196S mutant. The evolution of the catalytic efficiency of H196S between pH 5-7 might reflect the occupancy level of the metal-binding sites. These results underline the difficulty of deriving pK_a values for the catalytic groups when the affinity for the Zn cofactor is also pH-dependent. Indeed, at pH 5, at least 1 mM zinc is needed to saturate the enzyme and yield optimal catalytic activity. At pH 7, the k_{cat}/K_m values directly reflect the $[Zn]/[E]$ ratio. At 100 μ M Zn, two zinc ions are bound, and the catalytic efficiency increases by a factor of two when compared with the mono-zinc enzyme present at Zn concentrations $<5 \mu$ M (Table 4).

Among the mutants with modified active-site ligands, H196S was one of the most affected. In order to explain this loss of activity, a pH-rate profile was obtained, as in a previous study made on the wild-type enzyme by Bounaga et al. [18]. A feature of the pH-rate profile for the wild-type enzyme is the slope of 2 on the acidic 'limb', which was attributed to the ionization of two catalytically important groups that cannot be distinguished between with average pK_a values of 5.2. Interestingly, the H196S mutant appears to separate these two pK_a values, so that one remains close to 5.2 while the other is shifted to a higher pK_a of approx. 7 (Figure 2b). The latter presumably corresponds to the zinc-bound water, modified by the replacement of one of the zinc ligands. The lower pK_a probably represents that of Asp¹²⁰. The increase in the pK_a of the zinc-bound water corresponds to a more nucleophilic 'hydroxide-ion' because of the reduced effective positive charge on the metal ion. The observed reduced maximum catalytic efficiency of the H196S mutant at pH values above its pK_a therefore implies that, in addition to its effect on the pK_a of the metal-bound water, the zinc ion plays an important role as a Lewis acid, polarizing the β -lactam carbonyl group and stabilizing the development of negative charge on the carbonyl oxygen in the tetrahedral intermediate.

At basic pH (> 7), the limbs of the curves for the wild-type and H196S proteins were similar. A value of pK_a of 9.5 can be computed in both cases, although for the mutant there may be an indication of a slightly lower pK_a . The loss of catalytic efficiency cannot be attributable to the absence of zinc ions in the catalytic site, but to the variation of the ionization state of an essential residue that remains to be identified.

The situation is more complicated in the acidic pH range. The variation in the k_{cat}/K_m value at $pH < 7$ is dependent not only on the pK_a value of residues involved in the active site, but also on the $[Zn]/[E]$ ratio. The replacement of a histidine ligand of zinc with serine may substitute O for N co-ordination. There would then be two potentially ionizable sites: the proton from the co-ordinated serine, or that of bound water (Scheme 1). Hence the nucleophilicity of the zinc-bound hydroxide ion could be severely affected when compared with the wild-type enzyme. In contrast, substitution of the histidines with alanines or other amino acids with side chains with no metal-ion binding ability might result in a replacement of the altered Zn ligand by a water molecule and/or in a decreased affinity for the metal. The structure of the 'first' binding site is not symmetrical. The distances between the histidines and the zinc ion decrease in the following order: H116 $>$ H118 \geq H196. Interestingly, replacement of His¹¹⁶ with serine had a lesser effect on the catalytic properties of BcII when compared with the substitution of His¹¹⁸ and His¹⁹⁶. The catalytic efficiency of the mutants was not restored to the wild-type level by addition of zinc to the reaction mixture. The affinity for zinc was not significantly altered by the mutations, and each mutant could still bind two zinc ions. The affinity of mutant enzymes for the first metal ion appears to remain very high, since several dialyses against metal-free water ($[Zn^{2+}] \approx 20$ nM) did not impede its binding. The absence of one of the histidine side chains opens the active site, and creates a higher degree of flexibility for the substrate inside the catalytic site. This hypothesis could explain why the K_m values for the histidine mutants are so high. The difference in activity among the three histidine mutants has yet to be fully elucidated. However, modelling studies are in progress to assess further the substrate binding and the catalytic mechanism of the wild-type enzyme and the different mutants.

Scheme 1



Carbonic anhydrase contains a His_3 zinc polyhedron, which is comparable with the 'first' binding site of BcII. Removal of any one of the histidine ligands of carbonic anhydrase affects zinc binding, increases the rate constant for zinc dissociation and decreases the catalytic efficiency [29]. For BcII, substitution of the histidine residues modifies the activity of the enzyme, but apparently not its zinc-binding capability. These data suggest that the presence of a 'second' binding site for the metal influences the properties of the 'first' binding site.

Analysis of the second binding site

The second binding site of BcII comprises the Asp¹²⁰, Cys²²¹ and His²⁶³ residue side chains.

C221A and C221S

The substitution of the cysteine residue with alanine was studied previously by Paul-Soto et al. [13]. The mono- and the di-Zn forms of the C221A mutant exhibited different kinetic properties with respect to benzylpenicillin and nitrocefin, suggesting a role for the cysteine residue in efficient hydrolysis by the mono-Zn species, but not by the bi-nuclear enzyme. Indeed, an increased value of the catalytic efficiency, mainly due to a higher k_{cat} , was correlated with the zinc ion content of the protein. The data obtained with C221A and C221S with respect to one carbapenem (imipenem) and first- and third-generation cephalosporins (cephaloridine and cefotaxime) are in good agreement with these previous data. X-ray crystallographic data on the C168S mono-zinc form obtained at pH 5.6, in the presence of 100 μ M

dithiothreitol and 1 mM Zn(II), indicated that only one zinc-binding site was occupied [27]. The overall folding of the mutant was similar to that of the wild-type enzyme, with the side chain of Ser²²¹ occupying the same position as Cys²²¹ in the wild-type enzyme. Nevertheless, the distance between Zn-1 and water-1 was increased. These data support the hypothesis that a hydrogen bond is formed between Cys²²¹ and the Zn-bound water in the mono-zinc form of BcII. The replacement of the cysteine with a serine might impede this interaction because of the shorter C-O bond length compared with C-S, in spite of the better hydrogen-bonding properties of oxygen relative to sulphur. Hence the distance between the catalytic water molecule and the zinc ion will increase due to the reduction of the charge dispersal. As observed for the BcII cysteine mutants, the absence of the thiol group in the CcrA C221S and IMP-1 C221A, C221S and C221D mutants decreased drastically the catalytic efficiency of the enzymes in the absence of zinc [25,26]. The effect of zinc addition was not studied with the CcrA C221S mutant. However, in the case of the IMP-1 C221S mutant, Haruta et al. [26] have observed a different behaviour from that of the BcII mutant: the catalytic efficiency increased in the presence of zinc in the buffer, but this was mainly due to the decrease in the K_m value rather than an increase in k_{cat} . The [Zn]/[E] ratio of that mutant was measured only in zinc-free buffer. A different behaviour was also observed with the IMP-1 C221A mutant, for which the increase in the catalytic efficiency was due to both an increase in k_{cat} and a decrease in K_m .

D120N

The replacement of Asp¹²⁰ with an asparagine is another substitution that drastically affects the activity, but not the zinc-binding capability, of the enzyme. The [Zn]/[E] ratio for the D120N mutant is similar to that determined for the wild-type enzyme. Nevertheless, even with a high zinc concentration, the catalytic efficiency of the enzyme is more-than-100-fold lower with nitrocefm, benzylpenicillin and imipenem. Our data identify Asp¹²⁰ as an essential residue for the catalytic activity, but not for zinc binding. In the model proposed by Bounaga et al. [18], it was suggested that this residue had the role of a general base in the reaction catalysed by the mono-zinc form. The CcrA D120V and D120N mutants did not bind two zinc ions [15,25], which indicates that this residue could be essential for zinc binding by this enzyme. Nevertheless, substitution of the aspartate residue with a cysteine or a serine yielded an enzyme with a behaviour similar to that of the BcII D120N mutant [28]. These mutants were able to bind two zinc equivalents, and exhibited a large decrease of activity. These data add weight to the hypothesis that Asp¹²⁰ has a role in the catalytic process. In wild-type CcrA, Asp¹²⁰ might participate in the orientation of the water/hydroxide during the nucleophilic attack of the β -lactam ring, and also in the orientation of the second water molecule involved in the conversion of the apparent intermediate formed with this enzyme into free enzyme and product. This residue may assume a similar function in the di-zinc BcII.

H263S

Finally, the H263S substitution led to a weakly active enzyme. As observed for the hydrolysis of benzylpenicillin and nitrocefm by the H118S and H196S mutants, the K_m value strongly increased, whereas the k_{cat} value decreased by more than one order of magnitude. The catalytic efficiency of H263S towards nitrocefm and cefotaxime is dependent on the zinc ion concentration. Surprisingly, there is no apparent correlation between an increasing [Zn]/[E] ratio and the modification of the catalytic efficiency of the mutant. We found that only one equivalent of zinc ion was bound to the mutant in the presence of 100 μ M zinc. Nevertheless, EXAFS experiments performed at a zinc ion concentration of 1 mM indicated that the mutant can bind two zinc ions.

In the mono-zinc enzyme, both sites are almost equally occupied [13]. The addition of a second metal ion will yield two different species: the Zn₁ and Zn₂-BcII. The increase in the total activity observed in the wild-type enzyme will be due to the presence of the di-zinc enzyme, which is more active than the mono-zinc BcII. The mutation of the residue of the 'second' binding site led to a mono-zinc species that is poorly active. These data indicate that the catalytic mechanism, of the mono- and di-metallic species are different.

CONCLUSIONS

A large proportion of bi-nuclear metalloproteins, such as metallo- β -lactamases, glyoxalase II and aminopeptidase, appear to utilize a 'hydroxide ion', arising from a bridging water molecule in the nucleophile attack of the substrate. For glyoxalase II and aminopeptidase, an aspartic or glutamic acid residue has been proposed to hydrogen-bond with the hydroxide and thereby ensure a correct positioning of the nucleophile in the active site [30,31]. The function of the other metal ligands in the catalytic process is still poorly understood. The characterization of site-directed mutants pertaining to the different metal ligands shows that both sites are equally important for the catalytic activity of the mono- and di-zinc forms of BcII. At low Zn concentrations (0.4 μ M) and at neutral pH, all the mutant proteins contained one Zn ion. This suggests that the dissociation constant of the mono-zinc species has not been drastically increased by the mutations, a hypothesis which has been confirmed by titration experiments [32]. The binding of the second zinc ion might be somewhat more perturbed, and is certainly strongly increased in the case of H236S. With the exception of the Cys²²¹ mutants, the activity of all the modified proteins was only marginally increased by the binding of the second Zn ion, a behaviour similar to that found for the wild type. In contrast with the binding of the zinc cofactors, the activity was strongly influenced by all the mutations, again with the exception of the di-zinc form of the Cys²²¹ mutants. Interestingly, the disappearance of His²⁶³, a ligand of the second site, is more deleterious to the activity

than that of His¹¹⁶ and His¹¹⁸, which contribute to the 'first' binding site. Thus, the integrity of all six Zn ligands appears to be essential for optimal activity, even when possible modifications of the Zn binding constants are compensated for by the increased external Zn concentrations. These results underline the importance of both zinc-binding sites in the activity of the mono-zinc form, and the influence of the nature of the ligands on the catalytic efficiency of the Zn cofactors.

Acknowledgement

This work was supported in part by a grant from the European Union (grant ERB3512-Ic15-CT98-0914) as part of the Training and Mobility of Researchers Program and, by the Belgian Program Pôles d'Attraction Interuniversitaire initiated by the Belgian State, Prime Minister's Office, Services Fédéraux des Affaires Economiques, Techniques et Culturelles (PAI P4/03). D.d. S. and C. B. are Fellows of the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA; Brussels, Belgium). The AAS measurements were performed by the Laboratoire de la Santé et de l'Environnement, Institut Malvoz de la Province de Liège, Liège, Belgium.

REFERENCES

- Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O. and Frère, J.-M. (2001) *Antimicrob. Agents Chemother.* 45, 660-663
- Hussain, M., Carlino, A., Madonna, M. J. and Lampen, J. O. (1985) Cloning and sequencing of the metallothioprotein beta-lactamase II gene of *Bacillus cereus* 569/H in *Escherichia coli* J. *Bacteriol.* 164, 223-229
- Rasmussen, B. A., Gluzman, Y. and Tally, F. P. (1990) Cloning and sequencing of the class B beta-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. *Antimicrob. Agents Chemother.* 34, 1590-1592
- Yang, Y., Rasmussen, B. A. and Bush, K. (1992) Biochemical characterization of the metallo-beta-lactamase CcrA from *Bacteroides fragilis* TAL3636. *Antimicrob. Agents Chemother.* 36, 1155-1157
- Laraki, N., Franceschini, N., Rossolini, G. M., Santucci, P., Meunier, C., de Pauw, E., Amicosante, G., Frère, J. M. and Galleni, M. (1999) Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-beta-lactamase IMP-1 produced by *Escherichia coli*. *Antimicrob. Agents Chemother.* 43, 902-906
- Laraki, N., Galleni, M., Thamm, I., Riccio, M. L., Amicosante, G., Frère, J. M. and Rossolini, G. M. (1999) Structure of In31, a blaIMP-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. *Antimicrob. Agents Chemother.* 43, 890-901
- Senda, K., Arakawa, Y., Nakashima, K., Ito, H., Ichiyama, S., Shimokata, K., Kato, N. and Ohta, M. (1996) Multifocal outbreaks of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum beta-lactams, including carbapenems. *Antimicrob. Agents Chemother.* 40, 349-353
- Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N. and Ohta, M. (1996) PCR detection of metallo-beta-lactamase gene (blaIMP) in Gram-negative rods resistant to broad-spectrum beta-lactams. *J. Clin. Microbiol.* 34, 2909-2913
- Carfi, A., Duée, E., Galleni, M., Frère, J.-M. and Dideberg, O. (1998) 1.85 Å resolution structure of the zinc (II) beta-lactamase from *Bacillus cereus*. *Acta Crystallogr. Ser. D* 54, 313-323
- Concha, N. O., Rasmussen, B. A., Bush, K. and Herzberg, O. (1996) Crystal structure of the wide-spectrum binuclear zinc beta-lactamase from *Bacteroides fragilis*. *Structure* 4, 823-836
- Concha, N. O., Janson, C. A., Rowling, P., Pearson, S., Cheever, C. A., Clarke, B. P., Lewis, C., Galleni, M., Frère, J. M., Payne, D. J., Bateson, J. H. and Abdel-Meguid, S. S. (2000) Crystal structure of the IMP-1 metallo beta-lactamase from *Pseudomonas aeruginosa* and its complex with a mercaptocarboxylate inhibitor: binding determinants of a potent, broad-spectrum inhibitor. *Biochemistry* 39, 4288-4298
- Carfi, A., Pares, S., Duée, E., Galleni, M., Duez, C., Frère, J. M. and Dideberg, O. (1995) The 3-D structure of a zinc metallo-beta-lactamase from *Bacillus cereus* reveals a new type of protein fold. *EMBO J.* 14, 4914-4921
- Paul-Soto, R., Bauer, R., Frère, J. M., Galleni, M., Meyer-Klaucke, W., Nolting, H., Rossolini, G. M., de Seny, D., Hernandez-Valladares, M., Zeppezauer, M. and Adolph, H. W. (1999) Mono- and binuclear Zn²⁺-beta-lactamase. Role of the conserved cysteine in the catalytic mechanism. *J. Biol. Chem.* 274, 13242-13249
- Baldwin, G. S., Galdes, A., Hill, H. A. O., Smith, B. E., Waley, S. G. and Abraham, E. P. (1978) Histidine residues of zinc ligands in beta-lactamase II. *Biochem. J.* 175, 441-447
- Crowder, M. W., Wang, Z., Franklin, S. L., Zovinka, E. P. and Benkovic, S. J. (1996) Characterization of the metal-binding sites of the beta-lactamase from *Bacteroides fragilis*. *Biochemistry* 35, 12126-12132
- Bicknell, R. and Waley, S. G. (1985) Cryoenzymology of *Bacillus cereus* beta-lactamase II. *Biochemistry* 24, 6876-6887
- Bicknell, R., Schaffer, A., Waley, S. G. and Auld, D. S. (1986) Changes in the coordination geometry of the active-site metal during catalysis of benzylpenicillin hydrolysis by *Bacillus cereus* beta-lactamase II. *Biochemistry* 25, 7208-7215
- Bounaga, S., Laws, A. P., Galleni, M. and Page, M. I. (1998) The mechanism of catalysis and the inhibition of the *Bacillus cereus* zinc-dependent beta-lactamase. *Biochem. J.* 331, 703-711
- Wang, Z., Fast, W. and Benkovic, S. J. (1999) On the mechanism of the metallo-beta-lactamase from *Bacteroides fragilis* *Biochemistry* 38, 10013-10023
- Murphy, B. P. and Pratt, R. F. (1989) Evidence for an oxyanion hole in serine beta-lactamases and oo-peptidases. *Biochem. J.* 258, 765-768
- Sutton, B. J., Artymiuk, P. J., Cordero-Borboa, A. E., Little, C., Phillips, D. C. and Waley, S. G. (1987) An X-ray-crystallographic study of beta-

lactamase II from *Bacillus cereus* at 0.35 nm resolution. *Biochem. J.* 248, 181-188

- 22 Lim, H. M., Iyer, R. K. and Pène, J. J. (1991) Site-directed mutagenesis of dicarboxylic acids near the active site of *Bacillus cereus* 5/B/6 beta-lactamase II. *Biochem. J.* 276, 401-404
- 23 Lim, H. M. and Pène, J. J. (1989) Mutations affecting the catalytic activity of *Bacillus cereus* 5/B/6 beta-lactamase II. *J. Biol. Chem.* 264, 11682-11687
- 24 De Meester, F., Boris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J. M. and Waley, S. G. (1987) Automated analysis of enzyme inactivation phenomena. Application to beta-lactamases and DD-peptidases. *Biochem. Pharmacol.* 36, 2393-2403
- 25 Yang, Y., Keeney, D., Tang, X., Canfield, N. and Rasmussen, B. A. (1999) Kinetic properties and metal content of the metallo-beta-lactamase CcrA harboring selective amino acid substitutions. *J. Biol. Chem.* 274, 15706-15711
- 26 Haruta, S., Yamaguchi, H., Yamamoto, E. T., Eriguchi, Y., Nukaga, M., O'Hara, K. and Sawai, T. (2000) Functional analysis of the active site of a metallo-beta-lactamase proliferating in Japan. *Antimicrob. Agents Chemother.* 44, 2304-2309
- 27 Chantalat, L., Duée, E., Galleni, M., Frère, J.-M. and Dideberg, O. (2000) Structural effects of the active site mutation cysteine to serine in *Bacillus cereus* zinc-beta-lactamase. *Protein Sci.* 9, 1402-1406
- 28 Yanchak, M. P., Taylor, R. A. and Crowder, M. W. (2000) Mutational analysis of metallo-beta-lactamase CcrA from *Bacteroides fragilis*. *Biochemistry* 39, 11330-11339
- 29 Kiefer, L. L. and Fierke, C. A. (1994) Functional characterization of human carbonic anhydrase II variants with altered zinc binding sites. *Biochemistry* 33, 15233-15240
- 30 Chevrier, B., D'Orchymont, H., Schalk, C., Tarnus, C. and Moras, D. (1996) The structure of the *Aeromonas proteolytica* aminopeptidase complexed with a hydroxamate inhibitor. Involvement in catalysis of Glu151 and two zinc ions of the cocatalytic unit. *Eur. J. Biochem.* 237, 393-398
- 31 Zang, T. M., Hollman, D. A., Crawford, P. A., Crowder, M. W. and Makaroff, C. A. (2001) *Arabidopsis* glyoxalase II contains a zinc/iron binuclear metal center that is essential for substrate binding and catalysis. *J. Biol. Chem.* 276, 4788-4795
- 32 de Seny, D., Heinz, U., Wommer, S., Kiefer, M., Meyer-Klauck, W., Galleni, M., Frère, J. M., Bauer, R. and Adolph, H. W. (2001) Metal ion binding and coordination geometry for wild type and mutants of metallo- β -lactamase from *Bacillus cereus* 569/H/9 (BcII): a combined thermodynamic, kinetic, and spectroscopic approach. *J. Biol. Chem.* 276, 45065-45078