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Role of Cys221 and Asnll6 in the zinc-binding sites of the *Aeromonas hydrophila* metallo-*β*-lactamase

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Abstract:

The CphA metallo-*β*-actamase produced by *Aeromonas hydrophila* exhibits two zinc-binding sites. Maximum activity is obtained upon binding of one zinc ion, whereas binding of the second zinc ion results in a drastic decrease in the hydrolytic activity. In this study, we analyzed the role of Asn116 and Cys221, two residues of the active site. These residues were replaced by site-directed mutagenesis and the different mutants were characterized. The C221S and C221A mutants were seriously impaired in their ability to bind the first, catalytic zinc ion and were nearly completely inactive, indicating a major role for Cys221 in the binding of the catalytic metal ion.

By contrast, the binding of the second zinc ion was only slightly affected, at least for the C221S mutant. Mutation of Asn116 did not lead to a drastic decrease in the hydrolytic activity, indicating that this residue does not play a key role in the catalytic mechanism. However, the substitution of Asnl 16 by a Cys or His residue resulted in an approximately fivefold increase in the affinity for the second, inhibitory zinc ion. Together, these data suggested that the first zinc ion is located in the binding site involving the Cys221 and that the second zinc ion binds in the binding site involving Asn116 and, presumably, His118 and His196.

Key words: Metallo-*β*-lactamases; *β*-lactam resistance; zinc-binding sites; enzyme kinetics; site-directed mutagenesis.

β-Lactamases play a key role in bacterial resistance to *β*-lactam antibiotics, because they inactivate these compounds by efficiently catalyzing the hydrolysis of the amide bond of the *β*-lactam ring. On the basis of their primary structures, *β*-lactamases were classified into four families, referred to as classes A, B, C and D. Enzymes of classes A, C and D are serine enzymes, whereas class B *β*-lactamases are metallo-enzymes requiring Zn^{2+} for their activity [1, 2]. The class B family was further divided into three subclasses, B1, B2 and B3[3]·

Although metallo-*β*-lactamases were first described more than 30 years ago [4], they were originally found only in bacterial species of low or no pathogenic potential and, for this reason, were considered for a long time as biochemical curiosities. This view has changed in the past few years, however, as metallo-*β*-lactamases encoded by mobile genetic elements started to emerge in major nosocomial pathogens, including *Pseudomonas aeruginosa,*some Enterobacteriaceae and Acinetobacter spp. [5, and references therein].

To date, the three-dimensional structures of four class B *β*-lactamases - all belonging to subclasses B1 or B3 - have been solved by X-ray crystallography [6-12]. These studies revealed that metallo-*β*-lactamases possess an overall conserved fold with two zinc-binding sites. However, the metal-binding sites exhibit some notable differences between the members of the two subclasses. For subclass B1 enzymes, such as the *Bacillus cereus β*-lactamase BcII, one zinc ion is tetrahedrally coordinated by three generally well-conserved histidine residues (His116, His118 and His196) and a water molecule. The other metal ion has a trigonal-bipyramidal coordination

involving Asp120, Cys221, His263 and two water molecules. A water/hydroxide molecule serves as a ligand for both metal ions [6, 8, 9]. Here, these two metal-binding sites will be referred to as the 'histidine' site and the 'cysteine' site, respectively. Interestingly, in the mononuclear BcII enzyme, the sole metal ion was shown to be located in the 'histidine' site [6], which led to the idea that the presence of a zinc ion in this site might be a prerequisite for the activity of metallo-β-lactamases.

For subclass B3 β-lactamases, the 'histidine' site is identical to that found in BcII. The second zinc ion is bound to Asp120, His121, His263 and to the nucleophilic water, which also forms a bridge between the two metal ions. The cysteine 221 is replaced by a serine. This latter residue does not interact directly with the zinc ion but with a second water molecule located in the active site, which may serve as a proton donor during the catalytic process [12].

As mentioned above, no three-dimensional structure has been solved for a subclass B2 enzyme. The CphA metallo-β-lactamase produced by *Aeromonas hydrophila* belongs to this subclass [3]. By contrast to the BCcII and the *Bacteroides fragilis* enzymes and, generally speaking, to most other metallo-β-lactamases, CphA can be considered as a strict mono-zinc enzyme, the di-zinc state of the protein exhibiting a much lower hydrolytic activity [13]. Its activity is restricted to carbapenems; penicillins and cephalosporins are poorly or not hydrolyzed at all [14,15, and this study]. Furthermore, the histidine residue found at position 116 in most metallo-β-lactamases is replaced by an asparagine [3, 16].

Here, we analyzed the role of the Asn116 and Cys221 residues of the CphA enzyme by site-directed mutagenesis. We present data indicating that the first metal ion binds in the 'cysteine' site, which means that the primary, catalytically relevant metal-binding site of subclass B2 β-lactamases might differ from that found in subclass B1 and B3 enzymes.

MATERIALS AND METHODS

Bacterial strains and vectors

Escherichia coli XL-1 Blue (Stratagene Inc., La Jolla, Calif.) and *E. coli* BL21 (DE3) plysS (Novagen Inc., Madison, Wis.) were used as hosts for the construction of the different expression vectors and for the production of β-lactamases, respectively. pUC20-CphA was used as plasmid template in the PÇR site-directed mutagenesis amplifications. The expression vector pET-9a (Novagen) was used for the construction of the T7-based expression plasmid.

Chemicals and antibiotics

The primers used for the mutagenesis were synthesized by Amersham Pharmacia Biotech (Uppsala, Sweden). Kanamycin was purchased from Merck (Darmstadt, Germany). Benzylpenicillin ($\Delta\varepsilon_{235}$ = -800 M^{-1} cm^{-1}) was from Rhône-Poulenc (Paris, France). Imipenem ($\Delta\varepsilon_{300}$ = -9000 M^{-1} cm^{-1}) was from Merck Sharp and Dohme Research Laboratories (Rahway, N.J.). Cephaloridin ($\Delta\varepsilon_{260}$ = -10000 M^{-1} cm^{-1}) was purchased from Sigma (St Louis, Mo.) and nitrocefin ($\Delta\varepsilon_{482}$ = -15000 M^{-1} cm^{-1}) from Uni-path Oxoid (Basingstoke, UK).

Site-directed mutagenesis

The Quick Change Site-Directed Mutagenesis kit (Stratagene) was used to introduce all the mutations. The primers designed for these experiments are listed in table 1.

To introduce the N116C mutation in the CphA gene, we started from the pUC20 vector containing the CphA gene harboring the N116S mutation. The AGC codon encoding the serine residue was changed into a TGC codon encoding a cysteine residue. For the C221A mutation, we started from the gene encoding the C221S mutation (in pUC20). The AGC codon encoding a serine residue was replaced by a GCT codon encoding an alanine. The introduction of the desired mutation and the absence of additional unwanted mutations was always verified by sequence analysis.

Protein expression and purification

The genes encoding the mutant proteins were cloned into pET9a using the *Bam*HI and *Nde*I restriction sites. The different vectors obtained were then introduced into the *E. coli* BL21 (DE3) plysS strain. Overexpression and purification of the mutant proteins were performed as described for the wild-type protein [13], with the following

modifications. The bacteria expressing the wild type and the N116S proteins were grown for 24 h at 37 °C, those expressing the N116C and C221S mutants were grown for 48 h at 28 °C and those expressing the N116H and C221A mutants were grown for 72 h at 18 °C. This was necessary because all mutants but N116S were produced in a non- soluble form at 37 °C. The integrity of the purified proteins was verified using electrospray mass spectrometry (MS) on a Micromass Q-TOF system (Manchester, UK).

***Table** 1: List of mutagenic primers used to generate mutants,*

Primers	Sequences
N116Sfor	5'-GAGGTGATCAACACCAGCTACCACACCGACC-3'
N116Srev	5'-GGTCGGTGTGGTAGCTGGTGTTGATCACCTC-3'
NU6Cfor	5'-GAGGTGATCAACACCTGCTACCACACCGACC-3'
N116Crev	5'-GGTCGGTGTGGTAGCAGGTGTTGATCACCTC-3'
N116Hfor	5'-GAGGTGATCAACACCCACTACCACACCGACC-3'
N116Hrev	5'-GGTCGGTGTGGTAGTGGGTGTTGATCACCTC-3'
C221Sfor	5'-GCAGGTGCTCTATGGCAACAGCATTCTCAAGGAGAAGCTGG-3'
C221Srev	5'-CCAGCTTCTCCTTGAGAATGCTGTTGCCATAGAGCACCTGC-3'
C221Afor	5'-GCAGGTGCTCTATGGCAACGCTATTCTCAAGGAGAAGCTGG-3'
C221Arev	5'-CCAGCTTCTCCTTGAGAATAGCGTTGCCATAGAGCACCTGC-3'

The modified bases are underlined. for, forward primer; rev, reverse primer.

Determination of kinetic parameters

Hydrolysis of the antibiotics was monitored by following the absorbance variation resulting from the opening of the β-lactam ring. For imipenem, benzylpenicillin, cephaloridin and nitrocefin, the wavelengths were 300, 235, 260 and 482 nm, respectively. When the hydrolytic activity of the studied protein was high enough, k_{cat} and K_m parameters were determined from the complete hydrolysis time courses [17]. Otherwise, Km values were determined as Ki's using imipenem as a reporter substrate, and k_{cat} or k_{cat}/K_m values were obtained from initial rates of hydrolysis.

All experiments were performed at 30 °C in 15 mM sodium cacodylate, pH 6.5.

Enzymatic measurements in the presence of increasing concentrations of zinc and determination of K_{D2}

Residual activity in the presence of increasing concentrations of zinc was measured at 30 °C in 15 mM sodium cacodylate, pH 6.5, as described for the wild- type enzyme [13]. For the higher concentrations of zinc, the metal-catalyzed hydrolysis of the antibiotic could not be neglected, and initial rates of hydrolysis recorded in the presence of the enzyme were corrected accordingly. When binding of the second zinc ion resulted in a complete loss of activity, the data were analyzed using equation 1 [13].

$$RA=[K_{D2}/([Zn] + K_{D2})]\cdot 100 \qquad (1)$$

where RA is the residual activity and K_{D2} the dissociation constant for the second zinc ion. When binding of the second zinc ion resulted in an increase in activity, equation 2 was used:

$$RA = [(K_{D2} + \alpha [Zn])/([Zn] + K_{D2})] \cdot 100 \qquad (2)$$

where α represents the ratio of the activity at saturating concentrations of zinc versus activity in the absence of added zinc (Act. [Zn]/Act. $[Zn]_0$)· Experimental data were fitted to equations 1 or 2 by nonlinear regression analysis with the help of the software GraFit (Erithacus Software, Harley, UK).

Inactivation of wild-type protein in the C221S preparation by cefoxitin

Cefoxitin at a final concentration of 1 mM was added to the C221S mutant preparation (in 15 mM sodium cacodylate, pH 6.5). The sample was incubated overnight at 4°C anf then dialyzed against 15 mM sodium

cacodylate pH 6.5 to remove the excess cefoxitin. At this point, the hydrolytic activity of the preparation against imipenem was measured and compared to that exhibited by the preparation before cefoxitin treatment, and the procedure was repeated until the activity remained unchanged upon cefoxitin treatment (typically three times) to make sure that me wild-time enzyme was completely inactivated.

Determination of the zinc content by inductively coupled plasma mass spectrometry

Protein samples were dialyzed against 15 mM sodium cacodylate, pH 6.5. Protein concentrations were then determined by standard colorimetric assays (BCA; Pierce, Rockford, Ill.) and zinc concentrations were measured by inductively coupled plasma mass spectroscopy (ICPMS) as previously described [13].

RESULTS

Mutation of Asn116

Asn116 was replaced by a Ser, a Cys and a His residue. The three mutants were expressed in *E. coli* and purified to homogeneity. The presence of the mutation was verified by MS. Within the experimental errors, all three mutants were found to exhibit the expected mass (25,164 vs 25,161 Da for the N116S mutant, 25,180 vs 25,177 Da for the N116C mutant and 25,210 vs 25,211 Da for the N116H mutant). The MS spectra of the N116S and N116C mutants also exhibited minor peaks of lower molecular weight, suggesting that these mutants were partially proteolyzed. The mass of these lower-molecular-weight species (24,778 Da for the N116S mutant and 24,484 and 24,300 Da for the N116C mutant) corresponded to proteins having lost four (for the N116S mutant) and seven and nine (for the N116C mutant) residues at the C terminus. However, the proportion of these degraded species was too low (less than 5% of the total amount of protein) to interfere with the analysis of these proteins.

The activity of the three mutants was measured against imipenem, benzylpenicillin and cephaloridin, three compounds representing the three major families of β-lactam antibiotics, namely carbapemens, penicillins and cephalosporins, respectively. The activity of the N116H mutant against other carbapenems and nitrocefin, a widely used chromogenic substrate, was also determined. Among the tested compounds, only the carbapenems can be considered as good substrates for the wild-type enzyme [14,15].

The enzymatic activity of the different mutants was first measured in the absence of added zinc, i.e. for free Zn^{2+} concentrations in the range of 0.15-0.30 μM, as measured by ICPMS (not shown). Under these conditions, the N116S, N116C and N116H mutants were all found to contain only one zinc ion, similar to the wild-type enzyme (not shown). The kinetic data are summarized in table 2. The activity of the N116S mutant against imipenem was only slightly reduced, while its activity against cephaloridin remained virtually undetectable. The effect of the mutation on the activity against benzylpenicillin was more difficult to assess, as the activity of the mutant against this compound was low and difficult to measure accurately. The N116C mutant was markedly less active against imipenem. Its activity against benzylpenicillin and cephaloridin remained low, but the latter compound was a significantly better substrate for this mutant than for the wild-type enzyme. Finally, the Nl 16H mutation led to a ~20-fold decrease in the activity against imipenem but the enzyme was still active against different carbapenems. The activity against cephaloridin, and to a lesser extent benzylpenicillin and nitrocefin, was significantly increased. As mentioned above, the mono-zinc form of the wild-type enzyme is fully active, whereas the di-zinc protein is characterized by a much lower hydrolytic activity. Zn^{2+} indeed behaves as a non-competitive inhibitor, i.e. the catalytic constant k_{cat} decreases but K_m remains unaffected [13]. The dissociation constant for the second zinc ion (K_{D2}) can be computed from the plot of activity versus Zn concentration using equation 1.A value of K_{D2} = 46 μM was found for the wild-type enzyme using this approach [13].

The Nl16S, N116C and Nl16H mutants were found to exhibit similar behaviors. In the presence of increasing concentrations of zinc, the activity of the three mutants against imipenem decreased (fig. 1). From these curves, we found K_{D2} values of 50 ± 4,9 ± 0.5 and 12 ± 1 μM for the N116S, N116C and N116H mutants, respectively. Surprisingly, the activity of the N116H mutant against benzylpenicillin and cephaloridin increased (up to about 5- and 2-fold, respectively) in the presence of added zinc (fig. 1). Analysis of the experimental curves using equation 2 led to K_{D2} values of 8.3 ± 0.4 and 9 ± 2 μM, in good agreement with that calculated on the basis of the imipenem data. In this case too, k_{cat} increased while K_m remained unaffected (table 2). This behavior was observed neither for the wild-type enzyme nor for the N116S and N116C mutants, at least for benzylpenicillin (the activity of these enzymes against cephaloridin was too low to allow these measurements; data not shown).

***Table 2**: Kinetic parameters of the wild-type enzyme (WT) and the mutants N116S, Nl 16C and N116H.*

Enzyme	Substrate	$k_{cat}(S^{-1})$	$K_m(\mu M)$	$k_{cat}/K_m(M^{-1}s^{-1})$
WT	imipenem	1200 ± 70	340 ± 30	$3.5 \times 10^6 \pm 4 \times 10^5$
	meropenem	3100 ±200	1340 ± 200	$2.3 \times 10^6 \pm 3 \times 10^5$
	panipenem	1300 ± 100	500 ± 100	$2.5 \times 10^6 \pm 4 \times 10^5$
	biapenem	>100	>1500	$6.7 \times 10^4 \pm 10^4$
	benzylpenicillin	0.03 ± 0.003	870 ± 70	35 ±7
	cephaloridin	< 0.006	~6000	<l
	nitrocefin	0.0028 ± 0.0001	1200 ± 200	2.5 ± 0.2
N116S	imipenem	240 ± 50	540 ± 130	$4.5 \times 10^5 \pm 10^5$
	benzylpenicillin	<0.01	1500 ± 200*	<7
	cephaloridin	N.D.	>5000*	<3
N116C	imipenem	19 ± 4	500 ± 70	$3.9 \times 10^4 \pm 1.3 \times 10^4$
	benzylpenicillin	0.03 ± 0.006	3800 ±700*	8 ± 2
	cephaloridin	0.14 ± 0.02	8000 ±1000*	17 ± 1
N116H	imipenem	150 ± 10	1400 ± 100	$1.1 \times 10^5 \pm 10^4$
	meropenem	27 ± 5	1300 ± 200	$2.1 \times 10^4 \pm 2 \times 10^3$
	panipenem	1600 ± 200	750 ± 100	$2.1 \times 10^6 \pm 3 \times 10^5$
	biapenem	>1900	>2000	$9 \times 10^5 \pm 10^5$
	benzylpenicillin	0.38 ± 0.05	910 ± 120	420 ± 70
	benzylpenicillin + 40 μM $ZnCl_2$	1.39 ± 0.02	810 ± 40	1700 ± 100
	cephaloridin	0.24 ± 0.01	950 ± 50	260 ± 20
	cephaloridin + 40 μM $ZnCl_2$	0.47 ± 0.03	800 ± 100	580 ± 100
	nitrocefin	0.087 ± 0.008	33 ± 8	2700 ± 900

* Measured as Ki.
Measurements were done at 30 °C in 15 mM sodium cacodylate, pH 6.5. N.D., not determined.

Mutation of Cys221

Cys221 was replaced by a Ser or an Ala residue. The purified proteins were analyzed by MS. For the C221S mutant, the expected mass of 25,177 Da was found. By contrast, analysis of the C221A mutant indicated the presence of three majors peaks of 24,346, 24,276 and 23,503 Da (for an expected mass of 25,156 Da), indicating that the mutant protein had lost 8, 9 and 15 residues at the C terminus. There was no degradation at the N terminus, as confirmed by N-terminal sequencing (not shown). Surprisingly, we found that cefoxitin treatment of the C221S preparation resulted in a significant decrease in the hydrolytic activity against imipenem. This was completely unexpected because cefoxitin inactivates the CphA *β*-lactamase via a covalent modification of the Cys221 side chain [18], and we anticipated that the C221S mutant would be insensitive to this compound. We therefore concluded that the mutant preparation was contaminated by traces of wild-type protein. Similar findings have been reported before, and were suggested to occur through a 'misreading' at the transcriptional and/or trans-lational level [19, and references therein]. In the present case, however, the wild-type enzyme could be completely inactivated by repeated treatments with cefoxitin (see Materials and methods). This resulted in a ~200-fold decrease in the hydrolytic activity of the preparation against imipenem (not shown). All the data reported here (e.g. in table 3) were obtained after wild-type-enzyme inactiva-tion.

Interestingly, the C221A preparation was not contaminated by wild-type protein, i. e. the hydrolytic activity of the preparation was insensitive to the action of cefoxitin. This may be due to the fact mat the Cys → Ala mutation implies the modification of all three bases of the codon (vs only one for the Cys → Ser mutation), making the accidental replacement of Ala by Cys less likely. The activity of the two mutants could only be measured against imipenem; their activity against benzylpenicillin and cephaloridin was simply undetectable. Even with imipenem, the activity was so low that all the measurements had to be corrected for the rate of spontaneous hydrolysis of the substrate. These results are summarized in table 3. The replacement of the cysteine 221 led to a drastic decrease (five to six orders of magnitude) in the kinetic parameters k_{cat} and k_{cat}/K_m for imipenem. These data were obtained in the absence of added zinc. Under these conditions, both mutants exhibited a zinc content significantly lower than 1 but, however, higher than 0 (Zn/enzyme = 0.28 and 0.39 for the C221S and the C221A mutants, respectively). We first thought that this reflected the partial filling of the first binding site. Since the free zinc concentration in the buffers in the absence of added zinc was ~0.15-0.30 μM (see above), this would have indicated that the dissociation constant for the first zinc ion was in the micromolar

range. Similarly, the activities reported in table 3 would have originated from the fraction of enzyme containing one zinc ion. To test this hypothesis, we measured the hydrolytic activity of the two mutants in the presence of free zinc concentrations in the range of 5-20 μM. We anticipated that the addition of zinc would lead to an increase in the activity of the two mutants as the result of the filling of the first metal-binding site. We found, however, that the addition of zinc had little or no effect on the hydrolytic activity of the two mutants (not shown). This suggests that the Zn/enzyme ratios reported above (0.28 and 0.39) reflected non-specific binding of the metal ion to the protein rather than the partial filling of the first metal-binding site. Therefore, the activities reported in table 3 for the two cysteine mutants were most likely the activities of the apoenzymes. Interestingly, the C221S mutant could be inhibited by high concentrations of zinc (fig. 1), which indicates that the second, inhibitory site was still capable of binding a zinc ion. Analysis of the inhibition curve led to a K_{D2} value of 130 ± 13 μM. By contrast, the activity of the C221A mutant remained unaffected by free zinc concentrations up to 500 μM (fig. 1).

To see if the cysteine mutants were capable of binding more than one zinc ion, we also attempted to measure the zinc content of these mutants by ICPMS in the presence of high concentrations of free zinc (e.g. 100-200 μM), but obtaining consistent results using this approach was very difficult. The reason for this is unclear, but the mutants may be simply less stable and/or less soluble at the concentrations that one has to reach to perform these measurements.

DISCUSSION

Mutations at position 116

For three reasons, the *A. hydrophila* enzyme is pretty unique amongst class B *β*-lactamases. First, it is a strict mono-zinc enzyme, the binuclear form exhibiting a much lower activity. Second, only carbapenems are significantly hydrolyzed. Third, the His 116 found in most other metallo-*β*-lactamases is replaced by an asparagine. This latter point led us to investigate the role of Asn116 in the binding of the metal and during catalysis. This residue was replaced by a serine, a small hydrophilic amino acid which could possibly play the role of a weak metal ligand. The asparagine was also substituted by a bistidine to mimic the metal-binding site found in most other metallo-*β*-lactamases. The asparagine was also substituted by a cysteine. In that case, we tried to investigate the possibility to graft a new metal-binding site in me-tallo-*β*-lactamase. The presence of a cysteine in position 116 might create a metal-binding site (two cysteine and one histidine) similar to that found in the human alcohol dehydrogenase [20].

The activity of the N116S mutant against imipenem was only slightly reduced, indicating that Asn116 has no direct role in the catalytic mechanism. Similarly, Asn116 does not seem to participate in the binding of the second metal ion, since the K_{D2} values for the mutant and the wild-type enzyme are virtually identical (50 vs 46 μM).

The N116H mutant was not quite as active as the wild-type enzyme against imipenem, but benzylpenicillin, cephaloridin and nitrocefin were significantly better substrates. Thus, by recreating the three-histidine site found in most metallo-*β*-actamases, the properties of the enzyme are changed toward a broader substrate profile. This is illustrated in table 4. For the N116H mutant, kcat/Km values for benzylpenicillin, cephaloridin and nitrocefin are only two to three orders of magnitude lower than the kcat/Km value for imipenem, compared to four to six orders of magnitude for the wild-type protein. However, the presence of an asparagine at position 116 is far from being the only feature explaining the narrow substrate profile of the CphA enzyme, as the activity of the N116H mutant against nitrocefin, benzylpenicillin and cephaloridin remains low. Remarkably, the di-zinc state of the N116H mutant is not only active against the two latter compdunds, but it is even more active man the mono-zinc form, suggesting that, for this mutant, imipenem, on the one hand, and benzylpenicillin and cephaloridin, on the other, are hydrolyzed via different mechanisms. Yet, the most interesting finding concerning this mutant is that the affinity for the second metal ion (characterized by K_{D2}) increased about fivefold, which strongly suggests that the His116 side chain can interact with this metal ion. Therefore, the Asn116 side chain (and also presumably those of His118 and His196) would point into the 'second,' low-affinity inhibitory binding site and, consequently, the metal ion in the mono-zinc enzyme would be located in the other binding site, i.e. the one presumably involving Asp120, Cys221 and His263. This, much more than the presence of an Asn residue at position 116, may explain the particularly narrow substrate profile of the enzyme.

The N116C mutant was markedly less active against imipenem than N116S, possibly because the significantly larger side chain of a cysteine is not easily accommodated in the active site. Its activity against benzylpenicillin and cephaloridin remained low, although, as for the N116H mutant, the latter compound was a significantly

better substrate than it is for the wild-type enzyme (tables 2,4). Importantly, the N116C mutant also shared with the N116H mutant an approximately fivefold higher affinity for the second metal ion, again suggesting that, similarly to the His116 side chain in the N116H mutant, the Cys116 side chain is long enough and has a strong enough propensity to bind metal ions to coordinate with the second zinc ion. In addition, these results suggested that we could not create a new metal-binding site in the enzyme. Nevertheless, a spectroscopic study of the mutant is currently being performed to verify the nature of the metal coordination center in the N116C mutant.

Table3: *Values of k_{cat}, and k_{cat}/K_m for the interaction between imipenem and the wild-type CphA enzyme (WT) and the C221S and C221A mutants.*

Enzyme	k_{cat} (s^{-1})	K_m(MM)	k_{cat}/K_m (M^{-1} s-1)
WT	1200 ± 70	340 ± 30	3,500,000 ± 400,000
C221S	0.008 ± 0.001	210 ± 50	38 ± 9
C221A	0.0035 ± 0.0003	450 ± 90	8 ± 3

For the Cys221 mutants, the kinetic parameters were determined by measuring initial rates of hydrolysis of imipenem (0-1200 μM). Enzyme concentrations were in the range of 5 to 15 μM. Experiments were performed at 30°C in 15 mM sodium cacodylate, pH 6.5.

Table 4: *kcat and kcat/Km values for the wild type protein (WT) and the Nl 16S, N116C and N116H mutants (from Table 2) expressed as the ratio of the parameter measured against imipenem vs. the same parameter measured against the considered antibiotic.*

Enzyme	Antibiotic	$k_{cat(IMI)}/k_{cat(antibiotic)}$	$K_{cat}/K_{m(IMI)}/k_{cat}/K_{m(antibiotic)}$
WT	imipenem	1	1
	benzylpenicillin	4×10^4	10^5
	cephaloridin	$>2 \times 10^5$	$>3.5 \times 10^6$
	nitrocefin	4.3×10^4	1.4×10^6
N116S	imipenem	1	1
	benzylpenicillin	2.4×10^4	6.4×10^4
	cephaloridin	ND	$> 1.5 \times 10^5$
N116C	imipenem	1	1
	benzylpenicillin	630	4.3×10^3
	cephaloridin	140	2.3×10^3
N116H	imipenem	1	1
	benzylpenicillin	400	260
	cephaloridin	600	420
	nitrocefin	1.7×10^3	1080

N.D., not determined.

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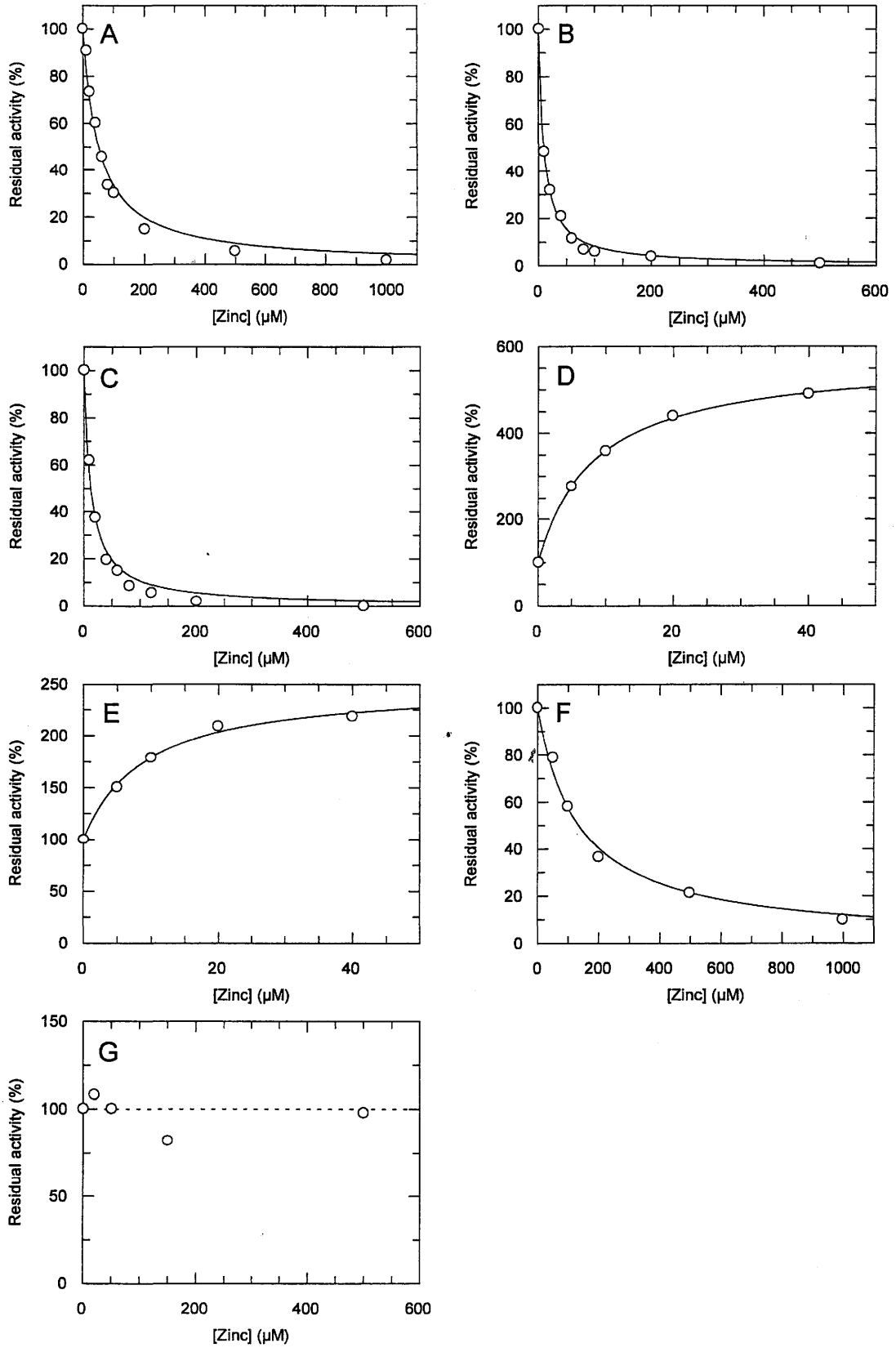
Figure 1 *: Residual activities measured in the presence of increasing concentrations of zinc. All experiments were performed at 30 °C in 15 mM sodium cacodylate pH 6.5. Mutants and substrates are as follows: N116S and imipenem (A), N116C and imipenem (B), N116H and imipenem (C), N116H and benzylpenicillin (D), N116H and cephaloridin (E), C221S and imipenem (F) and C221A and imipenem (G). The solid curves are the best fit using equation 1 (A-F) or 2 (D, E).*



Mutations at position 221

Both the C221A and the C221S mutants exhibited a zinc content lower than 1, indicating a drastic reduction of affinity for the first zinc ion (at least five or six orders of magnitude, considering that K_{D1} for the wild-type enzyme is 7 pM [21]). At the same time, at least for the C221S mutant, the affinity for the second metal ion is only very slightly affected (two to threefold). This strongly reinforces the hypothesis we proposed above, i.e. that the catalytic metal ion binds in the Asp120-Cys221 -His263 site and the inhibitory one in the Asnl 16-His118-His196 site. The C221A and C221S proteins also exhibited drastically reduced enzymatic activity, which at first sight can be attributed to the loss of the catalytic metal ion, although one cannot completely exclude that the Cys221 side chain also plays a more direct role in catalysis.

Interestingly, the activity of the C221A mutant is not affected by increasing concentrations of zinc (fig. 1), an observation which suggests that the Cys221 is the key to explaining why the mono-zinc enzyme is fully active and the di-zinc enzyme almost inactive. The exact mechanism of inhibition by excess zinc obviously remains unclear. One could envision for example that the Cys221 side chain participates directly in the catalysis in the monozinc enzyme, and is displaced (or at least made inaccessible) upon binding of the second metal ion. In the case of the C221S mutant, the fact that the hydrolytic activity still decreases upon addition of zinc suggests that the Ser221 side chain can to a certain extent play the role of the Cys221.

The modification of the Cys221 in the *B. fragilis* and in the *B. cereus* BcII *β*-lactamases also resulted in a dramatic decrease in the hydrolytic activity of the mononuclear enzyme, highlighting the important role of this residue in subclass B1 metallo-*β*-lactamases [22,23]. For the BcII enzyme, however, the activity was regained upon binding of the second metal ion. Whether or not this observation is relevant for the CphA *β*-lactamase is unclear, as the binuclear wild-type enzyme is almost devoid of activity.

The CphA metal-binding sites

The main findings of the present study are as follows: (i) the first zinc ion binds in the 'cysteine' site, and the Cys221 participates actively in its coordination; (ii) the second zinc ion binds in the 'histidine' site, but the Asnl 16 is involved neither in its coordination nor in the catalytic mechanism.

Although most metallo-*β*-lactamases are di-zinc enzymes under physiological conditions, they can usually function efficiently as mono-zinc enzymes [24, 25]. This suggests that the metal ion that binds first occupies the 'catalytic site whereas the second metal ion would have only a regulatory, or in some cases maybe only a structural, role. The CphA enzyme represents an extreme situation where full activity is obtained when the protein binds one zinc ion, the binding of the second one leading to an almost complete loss of activity. The first three-dimensional structure of a metallo-*β*-lac tamase was obtained for the BcII enzyme from *B. cereus* in its mono-zinc form [6]. In this case, the single metal ion was found to be located in the 'histidine' site, i.e. the site involving the histidine triad His 116, His118 and His196. It was therefore reasonable to assume that the presence of a zinc ion in the 'histidine' site is a prerequisite for the activity of metallo-*β*-lactamases. Our data provide a completely different view, with the catalytic metal ion being located in the 'cysteine' site in the CphA enzyme, and highlight the fact that, despite a certain structural homology, subclass B2 enzymes might function differently from subclasses B1 and B3 metallo-*β*-lac tamases.

The model describing a mono-zinc metallo-*β*-lactamase as a static species with the metal ion being located in a given binding site was questioned by more recent studies which suggest that the metal ion in the B. *cereus* BcII enzyme is distributed between the two sites [26]. However, whether such a model is relevant for the CphA enzyme, is not clear. Indeed, while this manuscript was in preparation, a detailed spectroscopic analysis of the CphA enzyme was published [27], According to the authors, the extended X-ray absorption fine-structure spectra are compatible with a model where the metal ion in the mononuclear, cadmium-substituted CphA enzyme is coordinated by one Cys, one His and one Asp (i. e. residues from the 'cysteine' site), without a detectable contribution of the 'histidine' site. By contrast, perturbed angular correlation of gamma-ray spectroscopy data suggest that the metal ion in the mononuclear, cadmium-substituted species might be distributed between the two sites, but with a strong preference (80%) for the 'cysteine' site. In any case, these data are in agreement with those reported here, and point toward the 'cysteine' site as being the main, catalytic metal-binding site in the CphA enzyme.

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