Published in: The Journal of Biological Chemistry (2005), vol. 280, iss.31, pp.28195-28202
Status: Postprint (Author's version)

Dramatic Broadening of the Substrate Profile of the *Aeromonas hydrophila* CphA Metallo-*β*-lactamase by Site-directed Mutagenesis*

Carine Bebrone‡§, Christine Anne‡§, Kris De Vriendt¶, Bart Devreese¶, Gian Maria Rossolini‖, Jozef Van Beeumen¶, Jean-Marie Frère‡, and Moreno Galleni‡**

From the ‡Centre d'Ingénierie des Protéines, Université de Liège, Allée de 6 Août B6, Sart-Tilman, 4000 Liège, Belgium, the ¶Laboratorium voor Eiwitbiochemie en Eiwitengineering, K. Ledeganckstraat 35, University of Gent, B-9000 Gent, Belgium, and the ‖Dipartimento di Biologia, Molecolare, Sezione di Microbiologia, Universita di Siena, Policlinico Le Scotte, 53100, Siena, Italy

Abstract: Among class B *β*-lactamases, the subclass B2 CphA enzyme is characterized by a unique specificity profile. CphA efficiently hydrolyzes only carbapenems. In this work, we generated site-directed mutants that possess a strongly broadened activity spectrum when compared with the WT CphA. Strikingly, the N116H/N220G double mutant exhibits a substrate profile close to that observed for the broad spectrum subclass B1 enzymes. The double mutant is significantly activated by the binding of a second zinc ion under conditions where the WT enzyme is non-competitively inhibited by the same ion.

Abreviations: WT, wild type; CD, circular dichroism

In vivo, class B *β*-lactamases (1) require one or two zinc ions as enzymatic cofactors. By efficiently catalyzing the hydrolysis of the *β*-lactam amide bond, these enzymes play a key role in bacterial resistance to this group of antibiotics. The metallo-*β*-lactamase family has been divided into three different subclasses, B1, B2, and B3, on the basis of sequence similarities (2, 3). The CphA metallo-*β*-lactamase produced by *Aeromonas hydrophila* belongs to subclass B2. It is characterized by a uniquely narrow specificity profile. CphA efficiently hydrolyzes only carbapenems and shows very poor activity against penicillins and cephalosporins, a behavior in contrast to that of metallo-*β*-lactamases of subclasses B1and B3, which usually exhibit very broad activity spectra against nearly all *β*-lactam compounds, with the exception of monobactams (4, 5). Moreover, in contrast to the BcII (*Bacillus cereus*) and CcrA (*Bacteroides fragilis*) enzymes belonging to the B1 subclass, and in general to most other metallo-*β*-lactamases, CphA exhibits a maximum activity as a mono-zinc enzyme. The presence of a Zn^{2+} ion in a second low affinity binding site non-competitively inhibits the enzyme with a K_i value of 46 μM at pH 6.5 (6). Recently, the structure of the mono-zinc CphA enzyme has been solved by x-ray crystallography (7). Similar to the known structures of metallo-*β*-lactamases of subclasses B1 (BcII (8), CcrA (9), IMP-I (10), BlaB (11)) and B3 (L1 (12) and FEZ-1 (13)), the x-ray structure of CphA highlights an *αββα* sandwich with two central *β*-sheets and α-helices on the external faces. The active site is located at the bottom of the *β*-sheet core. In agreement with previous spectroscopic results (14, 15) and site-directed mutagenesis studies (16), these structural data show that the sole Zn^{2+} ion resides in the Asp^{120}-Cys^{221}-His^{263} site of the *A. hydrophila* metallo-*β*-lactamase. In the di-zinc form of subclass B1, the zinc ions occupy both the His^{116}, His^{118}, and His^{196} and the Asp^{120}, Cys^{221}, and His^{263} sites (see Fig. 1). The histidine residue in position 116 in most metallo-*β*-lactamases is replaced by an asparagine residue in CphA (2, 17). This Asn-116 residue is not responsible for the narrow substrate profile of CphA, because the activity of the N116H mutant (where the three-histidine site found in most metallo-*β*-lactamases is recreated) against nitrocefin, benzyl-penicillin, and cephaloridine, although increased, remains rather low (16). Moreover, the K_D2 values are similar for the N116S mutant and the wild-type enzyme, indicating that Asn^{116} does not participate in the binding of the second metal ion.

* This work was supported in part by the Belgian Federal Government (Grant PAI P5/33), by the Fonds National de la Recherche Scientifique (FNRS) (Brussels, Belgium, Fonds de la Recherche Fondamentale et Collective (FRFC) Grants 2-4508.01 and 2-4524-03, Loterie Nationale 9.4538.03), and by the European Research Training Network (MEBEL contract HPTR-CT-2002-00264). The purchase of a Jasco J-810 spectropolarimeter was supported in part by a grant from the FRFC (contract number 2.4545.01). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipients of a Fonds National de la Recherche Scientifique dans l'Industrie et l'Agriculture (FNRS, Brussels, Belgium) and a Marie Curie fellowship, respectively.

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Among the strictly conserved residues (Table I), Arg^{121} is also present in most subclass B1 enzymes, with the exception of CcrA and IMP-1 where the arginine is replaced by a cysteine and a serine, respectively. In subclass B3, position 121 is occupied by a histidine residue that becomes a ligand for the second zinc ion (12, 13) (Fig. 1).

Cysteine 221 is also present in subclasses B1and B2. For CphA, its presence is essential to the interaction with the first zinc ion and for *β*-lactamase activity (3, 14-16). In subclass B1, substitution of the cysteine side chain yields a poorly active mono-zinc enzyme but does yield a di-zinc form nearly as active as the WT *β*-lactamases (18). In subclass B3, cysteine 221 is replaced by a serine, which does not interact directly with the second zinc ion but with a water molecule located in the active site and may serve as a proton donor during the catalytic process (12, 13).

The analysis of Fig. 1 and Table I indicated that we could progressively, by site-directed mutagenesis, replace the metal binding site motif of subclass B2 metallo-*β*-lactamase (Asn^{116}-X-His^{118}-*X*-Asp^{120} ... His^{196} ... Gly^{219}-Asn^{220}-Cys^{221} ... His^{263}) by those of subclass B1 (His^{116}-*X*-His^{118}-*X*-Asp^{120} ... His^{196} ... Gly^{219}-Gly^{220}-Cys^{221} ... His^{263}) or subclass B3 (His^{116}-*X*-His^{118}-*X*-Asp^{120}-His^{121}..His^{196} ... His^{263}), respectively.

These mutations strikingly broadened the CphA activity spectrum. Some mutants efficiently catalyzed the hydrolysis of penicillins and cephalosporins in addition to carbapenems.

FIG. 1: *Representation of the zinc binding sites of sub-class B1 (BCII), B2 (CphA), and B3 (Fez-1) β-lactamases. Amino acids that coordinate zinc ions are depicted.*

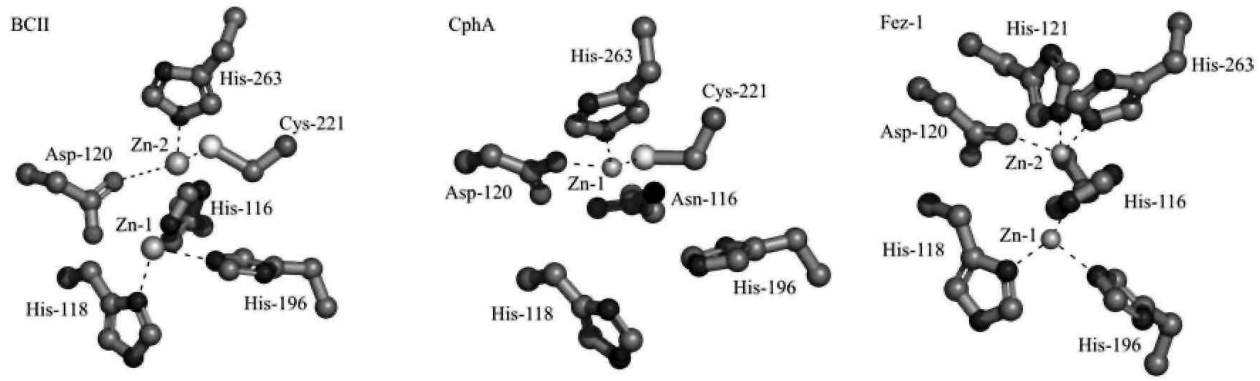

TABLE I: *Amino acid residues that are strictly conserved in the known subclass Bl, B2, and B3 zinc β-lactamases The residues in bold indicate the zinc ligands. The only exception is the 116 position in B3 where a Gln (Q) residue has been found in one case.*

Subclass	Positions																															
	56	73	79	84	103	116	117	118	120	121	123	134	142	150c[a]	183	193	195	196	197	199	203	206	217	219	220	221	232	244	263
B1				D		**H**		**H**	**D**		G		T			G		**H**		D	V	P	L	G	G	**C**	G	**W**	**H**
B2	D	V	G	G	S	N	T	H	**D**	R	G	A	T	I	G	G	A	H	T	D	V	P	L	G	N	**C**	G	Y	**H**
B3	G	L	G		G	**H/Q**	A	**D**	**H**			A		D	G		G	**H**	T							S			**H**

[a] Represents the position 150c in the amino acid sequence consensus of class B *β*-lactamases.

MATERIALS AND METHODS

Site-directed Mutagenesis

The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce all the mutations. The primers designed for these experiments are listed in Table II. The N220G mutant was constructed using pET9a/CphA WT as template. The double mutant N116H/N220G was constructed using pET9a/CphA N220G as template. Triple mutants N116H/R121H/ N220G and N116H/N220G/C221S were constructed from the N116H/ N220G mutant. The quadruple mutant N116H/R121H/N220G/C221S was made by adding mutation C221S to the N116H/R121H/N220G triple mutant. Finally, the triple mutant N116H/R121H/C221S was

obtained from the quadruple mutant by replacing Gly^{220} by the asparagine residue found in the wild-type enzyme.

Protein Expression and Purification

The genes encoding wild-type CphA and the mutant proteins were cloned into pET9a using the BamHI and Ndel restriction sites. The different vectors were then introduced into the *Escherichia coli* strain BL21(DE3) pLysS Star (In-vitrogen). Overexpression and purification of the mutant proteins were performed as described for the wild-type protein (6, 19) with the following modifications. Bacteria expressing the wild-type protein were grown for 8 h at 37 °C in 2XYT medium (Oxoïd, Backingstoke, UK), whereas those expressing the mutants were grown for 30 h at 18 °C in the same medium. The temperature was decreased to 18 °C because all the mutant proteins were produced mainly in a non-soluble form when the cultures were grown at 37 °C. The N116H/R121H/N220G/C221S mutant could never be produced in a soluble form even at 18 °C. The N116H/N220G/C221S and N116H/R121H/C221S mutants turned out to be very unstable and could not be purified in sufficient quantities for detailed characterization.

Determination of Kinetic Parameters

The hydrolysis of the 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 microcomputer via an RS232C serial interface. The wavelength and absorbance variations were those described by Matagne *et al.* (20, 21). Cells with 0.2- to 1-cm path lengths were used, depending on the substrate concentrations. When the K_m values of the studied enzymes were sufficiently high, the k_{cat} and K_m parameters were determined either under initial-rate conditions, using the Hanes linearization of the Henri-Michaelis-Menten equation, or by analyzing the complete hydrolysis time courses (22). Low and very high K_m values were determined as K_i values using imipenem or nitrocefin as reporter substrates. In the cases of low K_m values, the k_{cat} values were obtained from the initial hydrolysis rates measured at saturating substrate concentrations, and in the cases of high K_m values, k_{cat} was directly derived from *the* k_{cat}/K_m ratio.

All experiments were performed at 30 °C in 15 mM sodium cacodylate buffer, pH 6.5. Imipenem was from Merck Sharp and Dohme Research Laboratories (Rahway, NJ); benzylpenicillin, oxacillin, cephaloridine, and cefotaxime were from Sigma; and nitrocefin was from Unipath Oxoid (Basingstoke, UK).

TABLE II: *List of mutagenic primers used to generate mutants. The modified bases are underlined.*

Primers[a]	Sequences
N116Hfor	5'-CTGGAGGTGATCAACACCCACTACCACACCGACCG-3'
N116Hrev	5'-CGGTCGGTGTGGTAGTGGGTGTTGATCACCTCCAG-3'
N220Gfor	5' -CGAGCAGGTGCTCTATGGCGGCTGCATTCTCAAGGAG-3 '
N220Grev	5' -CTCCTTGAGAATGCAGCCGCCATAGAGCACCTGCTCG-3 '
R121Hfor	5'-CAACTACCACACCGACCACGCTGGCGGTAACGCC-3'
R121Hrev	5'-GGCGTTACCGCCAGCGTGGTCGGTGTGGTAGTTG-3'
TmR121Hfor	5'-CCACTACCACACCGACCACGCTGGCGGTAACGCC-3'
TmR121Hrev	5'-GGCGTTACCGCCAGCGTGGTCGGTGTGGTAGTGG-3'
TmC221Sfor	5'-GGTCCTCTATGGCGGCAGCATTCTCAAGGAGAAGC-3 '
TmC221Srev	5'-GCTTCTCCTTGAGAATGCTGCCGCCATAGAGGACC-3'
G220Nfor	5' -CGAGCAGGTGCTCTATGGCAACAGCATTCTCAAGGAG-3 '
G220Nrev	5' -CTCCTTGAGAATGCTGTTGCCATAGAGCACCTGCTCG-3 '

[a] For, forward primer; Rev: reverse primer.

Enzymatic Measurement in the Presence of Increasing Concentrations of Zinc and Determination of K_D2

Apparent k_{cat} and K_m values were measured in the presence of increasing concentrations of zinc at 30 °C in 15 mM sodium cacodylate, pH 6.5. When binding of the second zinc ion resulted in a complete loss of activity, the data were analyzed Equation 1 (6),

$$k_{cat}/k_{cat(0)} = [K_D2/([Zn] + K_D2)] \qquad \text{(Eq. 1)}$$

where $k_{cat}/k_{cat(0)}$ is the ratio between k_{cat} in the presence of zinc and k_{cat} in the absence of added zinc, and K_D2 is the dissociation constant for the second zinc ion. When binding of the second zinc ion resulted in an incomplete loss of activity or in an increase of activity, Equation 2 was used (16),

$$k_{cat}/k_{cat(0)} = [(K_D2 + \alpha[Zn])/([Zn] + K_D2)] \qquad \text{(Eq. 2)}$$

where α represents the ratio of k_{cat} at saturating zinc concentration over k_{cat} in the absence of added zinc. Experimental data were fitted to Equations 1 or 2 by non-linear regression analysis with the help of Kaleida-Graph for Windows (KaleidaGraph™, version 3.5, Synergy Software).

Electron Spray Ionization-Mass Spectrometry and Metal Content Determination

Samples of wild-type and mutant CphA enzymes were equilibrated in 10 mM ammonium acetate buffer (pH 6.5) by centrifugal filtration prior to electron spray ionization-mass spectrometry. For each protein mass spectra were obtained under both denaturing and native conditions. Denaturation of the protein was performed by dilution of the protein in a 50:50 (v/v) water/acetonitrile mixture containing 0.1% formic acid to final concentrations of 2-5 pmol/μl. The native protein was diluted to 10-15 pmol/μl in 10 mM ammonium acetate.

All mass spectra were acquired on a Q-TOF1 mass spectrometer (Micromass, UK) equipped with a nanoelectron spray ionization source using gold/lead-coated borosilicate needles purchased from Protana (Odense, Denmark). Capillary voltage was set at 1250 V and cone voltage at 40 and 60 V for the denatured protein and native protein, respectively. Acquisition time was 3-5 min across an *mlz* range of 400-3000. The mass spectra were processed with MassLynx version 3.1 software of Micromass. The instrument was calibrated using a mixture of myoglobin and trypsinogen. The zinc content of each protein was derived from the mass difference between the native and denatured protein.

Determination of the Zinc Content Using ICP/MS

Proteins samples were dialyzed against 15 mM sodium cacodylate, pH 6.5. Protein concentrations were then determined by measuring the absorbance at 280 nm (ϵ^{280} = 38,000 $M^{-1}cm^{-1}$). Zinc contents were determined by inductively coupled plasma mass spectroscopy as previously described (6,16).

TABLE III: *Metal binding for wild-type CphA and mutants*

Protein	Assay conditions	Electrospray ionization-mass spectrometry			
		Mass of protein		No. of Zn^{2+} ions	ICP/MS Zn^{2+} content
		Calculated	Measured		
CphA WT			*Da*	1	1
	Native		25,253.53		
	Denatured	25,189	25,189.62		
N116H	Native		25,274.47	1	1
	Denatured	25,212	25,212.27		
N220G	Native		25,196.71	1	1
	Denatured	25,132	25,132.92		
N116H/N220G	Native		25,218.77	1	1
	Denatured	25,155	25,154.34		
R121H	Native		25,232.5 (40%)	1	2 (expected: 1.6)
			25,294.5 (60%)	2	
	Denatured	25,169	25,169.28		
N116H/R121H/N220G	Native		25,198.32 (60%)	1	1.7 (expected: 1.4)
			25,262.37 (40%)	2	
	Denatured	25,136	25,135.68		

TABLE IV: *Kinetic parameters of the WT, N116H, N220G, and N116H/N220G CphA enzymes Measurements were performed at 30 °C in 15 mM sodium cacodylate, pH 6.5. S.D. values are below 10%. For the double mutant, values in parentheses are those observed at saturating zinc concentrations. Values for the WT enzyme and N116H are from Vanhove et al. (16).*

Enzyme	Substrate	k_{cat}	K_m	k_{cat}/K_m
		s^{-1}	μM	$M^{-1}s^{-1}$
WT	Imipenem	1200	340	3,500,000
	Benzylpenicillin	0.03	870[a]	35
	Oxacillin	>l	>2,000	500
	Ampicillin	<0.0l	2,500[a]	<4
	Nitrocefin	0.008	1,300	6
	Cephaloridine	<0.006	6,000[a]	<l
	Cefotaxime	>0.0002	>100	2
N116H	Imipenem	150	1,400	110,000
	Benzylpenicillin	0.4	910	440
	Nitrocefin	0.09	33	2,700
	Cephaloridine	0.24	950	250
N220G	Imipenem	390	50	7,800,000
	Benzylpenicillin	0.06	530°	120
	Nitrocefin	0.008	400	20
	Cephaloridine	0.03	5,500°	5.5
	Cefotaxime	0.009	1,000°	9
N116H/N220G	Imipenem	16(8)	185 (170)	86,000 (47,000)
	Benzylpenicillin	3.3 (20)	150° (150)	22,000 (130,000)
	Oxacillin	10	480	21,000
	Ampicillin	0.8	800	1,000
	Nitrocefin	0.7 (2)	4(3.7)	175,000 (540,000)
	Cephaloridine	0.9 (11)	145(145)	6,200 (75,000)
	Cefotaxime	0.3 (4)	50 (50)	6,000 (80,000)

[a] Measured as K_i values.

TABLE V: *Initial rates of hydrolysis for the N116H/N220G and some β-lactam antibiotics. The concentration was 100 µM for each substrate. v_0 is expressed in micromoles of substrate hydrolyzed per minute and per mg of protein. The volume of reaction is 500 µl in each case.*

Antibiotics	v_0
	$\mu mol\ min^{-1}\ mg^{-1}$
Imipenem	6.8
Biapenem	1.2
Meropenem	4
Benzylpenicillin	1.6
Nitrocefin	0.8
CENTA	0.6
Cephalothin	1.4
Ceftriaxone	0.4
Cefaclor	0.4

Stability toward Chaotropic Agents

The stability of the different proteins was studied by fluorescence. The WT and mutant enzymes (0.05 mg/ml) were incubated for 16 h in the presence of increasing urea concentrations (0-8 M) at 4 °C. Fluorescence emission spectra of the enzymes were recorded at 20 °C with a PerkinElmer Life Sciences LS50B luminescence spectrometer using excitation and emission wavelengths of 280 and 333 nm, respectively.

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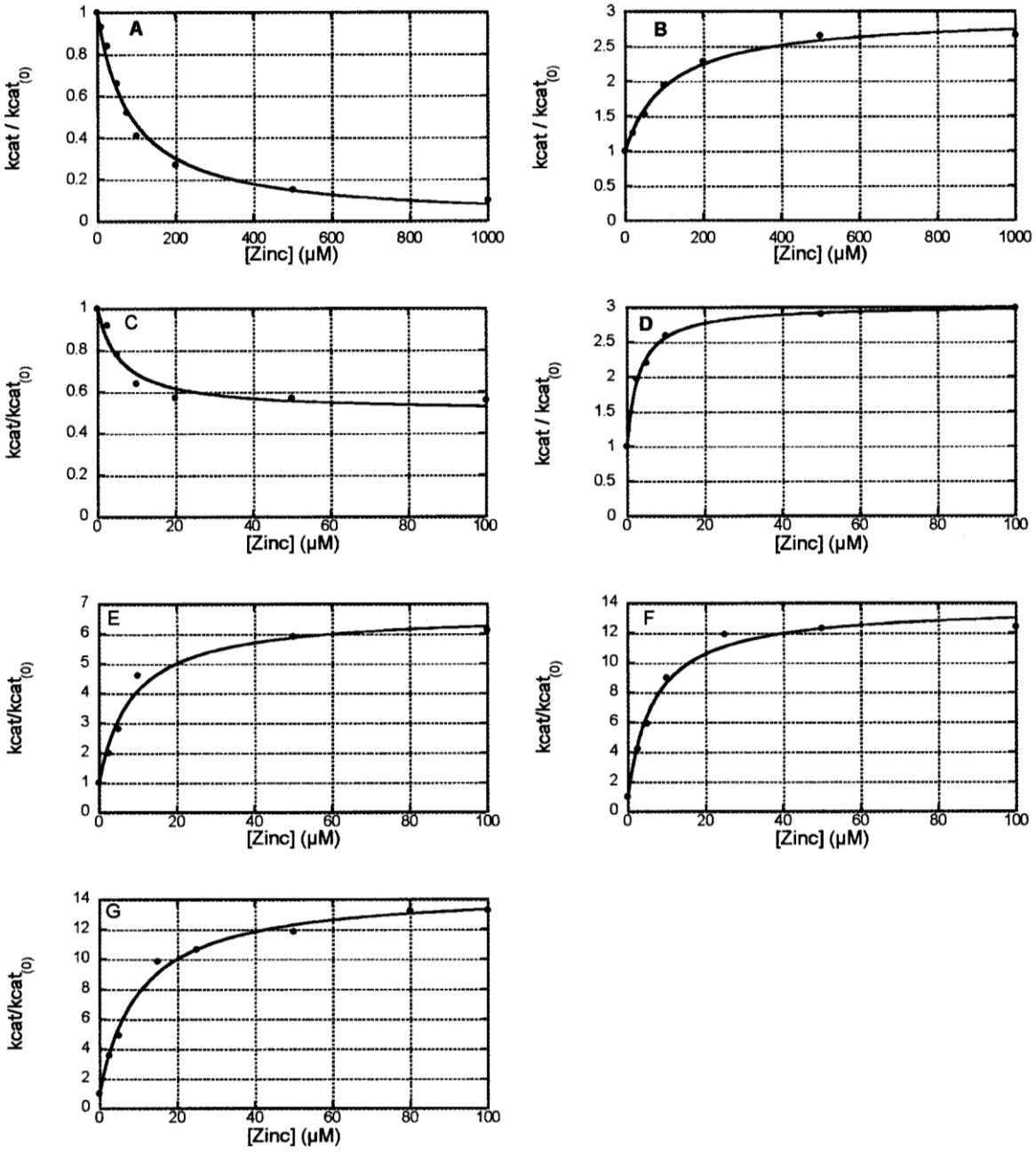
Circular Dichroism

CD spectra of the different enzymes (0.5 mg/ml) were obtained using a Jasco J-810 spectropolarimeter. The spectra were scanned at 20 °C with 1-nm steps from 200 to 250 nm (far UV) and from 250 to 310 nm (near UV).

FIG. 2: *Catalytic properties of the N220G and N116H/N220G mutants. Relative k_{cat} values ($k_{cat}/k_{cat(0)}$) measured in the presence of increasing concentrations of zinc. A, N220G and imipenem; B, N220G and nitrocefin; C, N116H/N220G and imipenem; D, N116H/N220G and nitrocefin; E, N116H/N220G and benzylpenicillin; F, N116H/N220G and cephaloridine; G, N116H/N220G and cefotaxime. The solid curves are the best fit using Equation 1 (A) or Equation 2 (B-G). All experiments were performed at 30 °C in 15 mM sodium cacodylate, pH 6.5. S.D. values are below 10%. The substrate concentrations were 100 µM with the exception of benzylpenicillin (500 µM).*



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RESULTS

From a B2 to a B1 Enzyme

The N220G and N116H/N220G mutants were expressed in *E. coli* BL21(DE3) pLysS Star and purified to homogeneity. The mass of the different proteins was verified by electrospray mass spectrometry. Within experimental errors, the mutants were found to exhibit the expected masses (25132.92 *versus* 25132 Da for N220G and 25154.34 *versus* 25155 for N116H/N220G, Table III).

The CD spectra of the mutants in the far UV indicated the same *a/β* ratio as for the wild-type enzyme. CD spectra in near UV and fluorescence emission spectra of the N116H/N220G mutant suggest a small conformational change in the protein tertiary structure (not shown).

The stability of the N220G mutant with urea as denaturat-ing agent is similar to that of the WT enzyme. Transitions between native and denaturated states occurred near 3 M urea. The N116H/N220G mutant is a little less stable than the WT enzyme and the N220G mutant, because the transition already occurred between 2 and 3 M urea. The enzymes were stored in 15 mM sodium cacodylate, pH 6.5, and in the presence of a free zinc concentration lower than 0.4 μM. Under these conditions, the N220G and N116H/N220G proteins contained one zinc ion per molecule as reported for the WT β-lactamase (Table III). At a free zinc concentration of 100 μM, inductively coupled plasma mass spectroscopy results show that the WT enzyme and the N116H/N220G double mutant bind 2 zinc ions per molecule, whereas the N220G mutant binds 1.7 zinc ions per molecule. The activity of the two mutants was measured in the absence of added Zn^{2+} (<0.4 μM) with various substrates representing the three major families of β-lactam antibiotics, namely carbapen-ems, penicillins, and cephalosporins (Table IV). The N116H substitution (16) modified the activity profile of CphA. The mutant was 20-fold less active against imipenem, but the k_{cat} values for benzylpenicillin, cephaloridine, and nitrocefin, although significantly increased, remained quite low so that imipenem remained the best substrate by two orders of magnitude or more.

The behavior of N220G CphA is not very different from that of the wild-type enzyme (Table IV). By contrast, the substrate profile of the N116H7N220G mutant is completely different. It no longer behaves as a strict carbapenemase. It hydrolyzes benzylpenicillin, oxacillin, ampicillin, nitrocefin, cephaloridine, cefuroxime, and cefotaxime, in addition to imipenem. When compared with the WT enzyme, the k_{cat} value of the latter decreased by 75-fold, but for all the other substrates, the K_m values decreased and the k_{cat} values increased (Table IV). Moreover, the N116H/N220G mutant efficiently catalyzes the hydrolysis of meropenem, biapenem, CENTA, cephalothin, cefaclor, and ceftriaxone (Table V).

As mentioned before, the mono-zinc form of the wild-type enzyme (observed in the presence of contaminating zinc concentration, *i.e.* ~0.4 μM) is active, and added zinc behaves as a non-competitive inhibitor resulting in negligible activity at high Zn^{2+} concentrations with a K_i *(K_D2)* value of 46 μM (6). With imipenem, the behavior of N220G was similar to that of the WT, but the K_D2 value increased to 86 ± 6 μM (see Fig. *2B).* By contrast, and despite a decrease of K_D2 to 5 ± 0.9 μM, the double mutant retained 50% of activity at saturating Zn^{2+} concentrations (Fig. 1C).

In both cases, the addition of Zn^{2+} did not affect the K_m values (Table VI). With the other substrates, and as already observed with the N116H mutant, the activity of the N116H7 N220G double mutant increased up to 14-fold in the presence of added zinc (Fig. 1, *D-G).* Analysis of the experimental curves using Equation 2 yielded values of 3 ± 0.24, 9 ± 1.8, 7 ± 1, and 9 ± 1 μM for the dissociation constant of the second zinc ion with nitrocefin, benzylpenicillin, cephaloridine, and cefotaxime as substrates, respectively.

In these cases too, only k_{cat} was affected, K_m being unaffected (Table VII). This behavior was also observed for N220G, at least for nitrocefin (the activities of this enzyme against benzylpenicillin, cephaloridine, and cefotaxime were too low to allow meaningful measurements), and K_D2 obtained from this curve using Equation 2 was 110 ± 13 μM (Fig. *2B*).

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TABLE VI: *Influence of zinc concentrations on k_{cat} and K_m values for N116H/N220G and imipenem S.D. values are below 10%.*

	[Zinc]	k_{cat}	K_m
	μM	s^{-1}	*μM*
Imipenem	<0.4	16	185
	1	14.4	190
	5	12.5	190
	10	10.4	160
	20	9	170
	50	8.7	160
	100	8.5	160
	1000	8	170

TABLE VII: *Influence of zinc concentrations on k_{cat} and K_m values for N116H/N220G and nitrocefin, benzylpenicillin, cephaloridine, and cefotaxime. S.D. values are below 10%.*

Antibiotics	Zinc	k_{cat}	K_m
	μM	s^{-1}	*μM*
Nitrocefin	<0.4	0.69	4
	2.5	1.36	3
	5	1.52	3.5
	10	1.79	3
	50	2.00	3.4
	100	2.06	3.7
Benzylpenicillin	<0.4	3.30	150
	100	20	150
Cephaloridine	<0.4	0.90	145
	100	11	145
Cefotaxime	<0.4	0.30	50
	100	4	50

TABLE VIII: *Kinetic parameters of the R121H and N116H/R121H/N220G mutants Values in parentheses are for the N116H/N220G enzyme in the absence of added zinc. Measurements were performed at 30 °C in 15 mM sodium cacodylate, pH 6.5. S.D. values are below 10%. Note that the k_{cat} values of the triple mutant versus imipenem and nitrocefin increase, respectively, 1.9- and 2.5-fold at zinc saturation without significant K_m modification.*

Enzyme	Substrate	k_{cat}	K_m	k_{cat}/K_m
			μ,M	
WT	Imipenem	1,200	340	3,500,000
	Benzylpenicillin	0.03	870[a]	35
	Nitrocefin	0.008	1300	6
	Cephaloridine	<0.006	6,000[a]	<l
	Cefotaxime	>0.0002	> 100	2
R121H	Imipenem	3.6	380	9,500
	Benzylpenicillin	0.11	11,000[a]	10
	Nitrocefin	0.003	10	300
	Cephaloridine	0.03	1,800°	17
	Cefotaxime	0.0017	245°	7
N116H/R121H/N220G	Imipenem	1.4(16)	63 (185)	22,000 (86,000)
	Benzylpenicillin	0.35 (3.3)	1,500° (150)	233 (22,000)
	Nitrocefin	0.5 (0.7)	85(4)	6,000 (175,000)
	Cephaloridine	0.2 (0.9)	250° (145)	800 (6,200)
	Cefotaxime	0.1 (0.3)	120° (50)	833 (6,000)

[a] Measured as K_i values.

Published in: The Journal of Biological Chemistry (2005), vol. 280, iss.31, pp.28195-28202
Status: Postprint (Author's version)

From a B2 to B3 enzyme

Only the R121H and N116H7 R121H/N220G mutants could be produced in *E. coli* and purified to homogeneity in sufficient quantities. Masses were 25169 Da for R121H and 25136 Da for N116H7R121H7N220G, in agreement with the theoretical values (Table III).

The far-UV CD spectra of the mutants are similar to that of the wild-type. The near-UV CD and fluorescence emission spectra of these mutants indicated small conformational modifications in the tertiary structure. The stabilities of the mutants in the presence of increasing concentrations of urea were a little lower than that of the WT. Transitions between native and denaturated states already occurred between 2 and 3 M.

FIG. 3: *Catalytic properties of the R121H and N116H/R121H/N220G mutants. Relatives k_{cat} values ($k_{cat}/k_{cat\ 0}$) measured in the presence of increasing concentrations of zinc. A, R121H and imipenem; B, N116H/R121H/N220G and imipenem; C, R121H and nitrocefin; D, N116H/ R121H/N220G and nitrocefin. The solid curves are the best fit using Equation 1 (A) or Equation 2 (B and D). All experiments were performed at 30 °C in 15 mM sodium cacodylate, pH 6.5. S.D. values are below 10%. The substrate concentrations were 100 µM.*

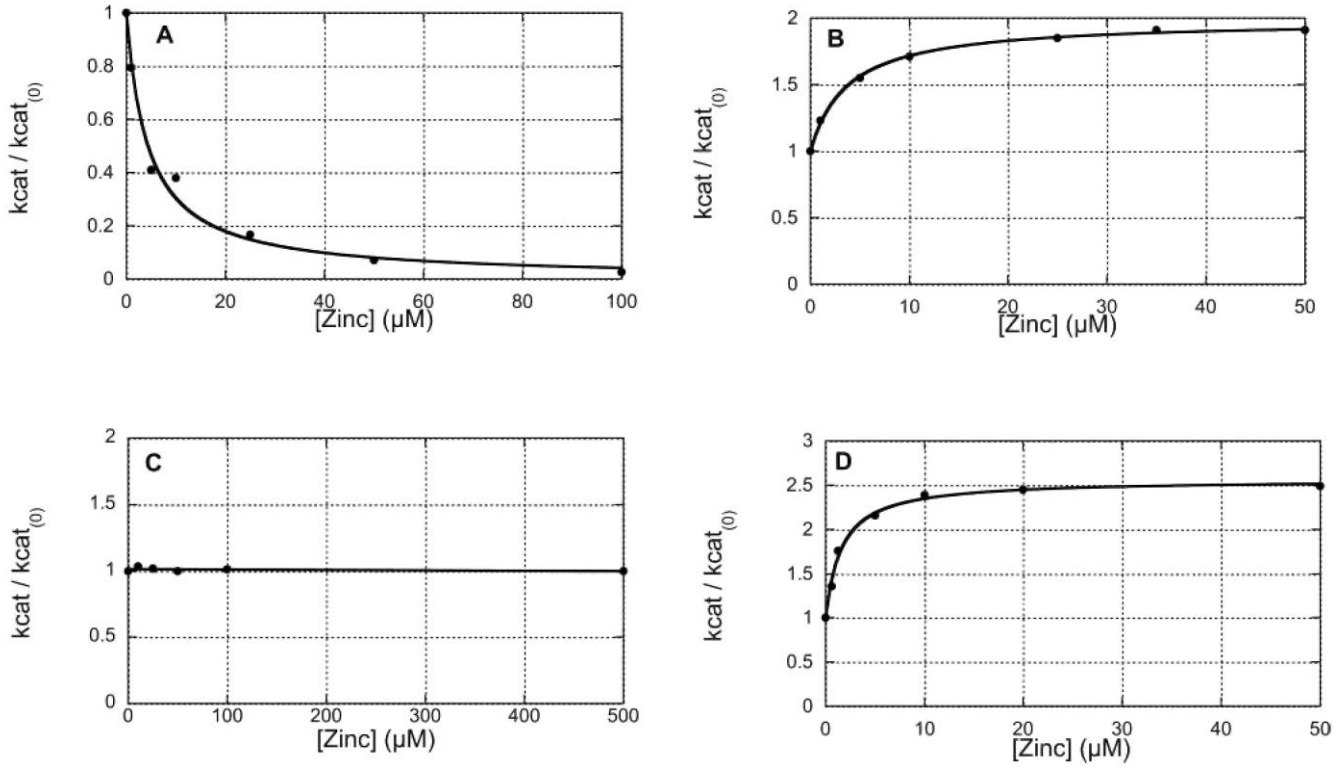

TABLE IX: *K_D2 values for the N220G and N116HIN220G mutants The values are derived from the data shown in Fig. 1.*

Mutant	Substrate	K_D2
		µM
N220G	Imipenem	86 ±6
	Nitrocefin	110 ± 10
N116H/N220G	Imipenem	5 ± 0.9
	Nitrocefin	3 ± 0.24
	Benzylpenicillin	9 ± 1.8
	Cephaloridine	7 ± 1
	Cefotaxime	9 ± 1

The activity of these two mutants was measured with imipenem, benzylpenicillin, nitrocefin, cephaloridine, and cefotaxime as substrates in the absence of added zinc, *i.e.* for a free zinc concentration below 0.4 μM (Table VIII). Under these conditions, a significant proportion of a di-zinc form was already present for both mutants, respectively, 60% for R121H and 40% for N116H/R121H/N220G, in contrast to the wild-type protein and to the N220G and N116H/N220G mutants, which were 100% mono-zinc under the same conditions (Table III). With imipenem, the R121H mutant was much less active than the wild-type, a loss of activity due to a 300-fold decrease of the k_{cat} value. The K_m value of benzylpenicillin significantly increased, whereas those of nitrocefin and cephaloridine decreased. The k_{cat}/K_m values increased significantly only for nitrocefin and cephaloridine but did so for different reasons, namely a decrease *of* K_m for the former and an increase of k_{cat} for the latter. The triple mutant exhibited a strong decrease of activity *versus* imipenem (due to a decrease of k_{cat}) and, as for the N116H/N220G mutant, a significant increase *versus* all other substrates due to increased k_{cat} values and, in the cases of nitrocefin and cephaloridine, significantly decreased K_m values. However, in all cases, the k_{cat}/K_m values of the triple mutant remained well below those of N116H/N220G.

The activity of the R121H mutant against imipenem decreased when the zinc concentration increased (Fig. 3A). Considering that in the absence of added zinc ($[Zn^{2+}] < 0.4$ μM), 60% of the R121H enzyme is already in a di-zinc form, K_D2 can be estimated to be below 1.0 μM. The activity of R121H with nitrocefin as substrate is independent of the zinc concentration (Fig. 3C). Surprisingly, the activity of the N116H/R121H/ N220G mutant with imipenem and nitrocefin as substrates increased (1.9- and 2.5-fold, respectively) in the presence of added zinc (Fig. 2, *B* and *D).* Considering that, in the absence of added zinc ($[Zn^{2+}] < 0.4$ μM), 40% of the N116H/R121H/ N220G enzyme was already in a di-zinc form, K_D2 can be estimated to be below 1.0 μM.

DISCUSSION

From a B2 to Bl Métallo-β-lactamase

On the basis of sequence alignments, the B2 characteristic motif of CphA has been modified to mimic that found in broad spectrum B1enzymes. The N116H mutant has already been studied by Van-hove *et al.* (16). Here, Asn^{220} was replaced by a glycine residue, and the N116H/N220G double mutant was constructed.

Although the k_{cat}/K_m values of the N220G mutant were somewhat increased with benzylpenicillin, nitrocefin, cephaloridine, and cefotaxime when compared with the WT protein, its activity *versus* imipenem also slightly increased. These results indicate that the Asn^{220} side chain does not have a significant role in the catalytic process and does not influence the residues involved in this process. The mutation results in a higher K_D2 value for the binding of the second zinc ion (Table IX). Titration of the apoenzyme[2] of N220G by Zn^{2+} also indicates that binding of the second zinc ion is more difficult than with the WT enzyme. The three-dimensional structure of the mono-zinc N220G mutant shows that the increased backbone mobility due to the N220G mutation alters the ability of Cys^{221} to coordinate the zinc. As a consequence, the zinc ion occupies two sites, the wild-type site and a new site in which the zinc ion presents a tetrahedral geometry with the triad Asp^{120}-Cys^{221}-His^{263}, the fourth ligand being Arg^{121} (7).

TABLE X: *Values of (k_{cat}/K_m antibiotic)/ (k_{cat}/K_m imipenem) for the CphA WT, N116/N220G, and N116H/R121H/N220G CphA mutants, subclass B1 (BcII, IMP-1, and VIM-2), and subclass B3 (L1 and FEZ-1) enzymes*

	k_{cat}/K_m (antibiotic)/k_{cat}/K_m (imipenem)								
	CphA (Subclass B2)				Subclass Bl			Subclass B3	
	WT	N116H/N220G [Zn] ≥ 0.4 μM	N116H/N220G [Zn] = 100 μM	N116H/R121H/N220G	BeII	IMP-1	VIM-2	L1	FEZ-1
Benzylpenicillin	<0.00l	0.23	2.5	0.01	3.75	0.5	1	0.27	0.55
Cephaloridin	<0.00l	0.07	1.4	0.04	0.16	2	0.7	0.13	0.08
Cefotaxime	<0.00l	0.07	1.5	0.04	6	0.3	1.5	3	12

With imipenem as substrate, the di-zinc form of N220G is completely devoid of activity as observed for the wild-type CphA. In contrast, with nitrocefin as substrate, the di-zinc form of N220G is more active than the mono-zinc form, whereas there is no influence of added zinc on the activity of the WT enzyme *versus* nitrocefin.

[2] K. De Vriendt, unpublished observation.

The N116H/N220G double mutant was not as active as the wild-type enzyme against imipenem, but several penicillins and cephalosporins were now found to be significantly hydro-lyzed. Thus, recreating the characteristic motif of the B1subclass clearly broadens the substrate profile. For this mutant, the k_{cat}/K_m values for benzylpenicillin, cephaloridine, and cefotaxime are of the same order as, or are only 1 order of magnitude lower than the k_{cat}/K_m value for imipenem, compared with 4-6 orders for the WT. The k_{cat}/K_m value for nitrocefin is even 2-fold higher, and this increases to 11-fold in the presence of added zinc (see below).

With N116H/N220G and as already observed for N116H (16), the di-zinc form is more active against benzylpenicillin, cephaloridine, cefotaxime, and nitrocefin than the mono-zinc form, contrary to what happens for imipenem. This suggests that imipenem on the one hand, and these latter compounds on the other, are hydrolyzed via slightly different mechanisms. However, the hydrolysis of imipenem by the N116H/N220G mutant is not totally inhibited by the binding of the second zinc ion, which underscores a significant residual activity of the di-zinc form with this substrate. For the latter, the k_{cat}/K_m values for benzylpenicillin, nitrocefin, cephaloridine, and cefotaxime are of the same order as, or are 1 order of magnitude higher than the k_{cat}/K_m value for imipenem (Table IV), and the k_{cat}/K_m values for all the tested substrates are of the same order of magnitude as, or are only 1 order lower than those determined for the subclass B1 BcII enzyme (5), which is considered to exhibit a broad activity spectrum. In the N116H/N220G mutant, the affinity for the second metal ion (characterized by K_D2) increased by 5- to 10-fold suggesting that the His^{116} side chain can interact with the zinc ion. This hypothesis was already proposed for the N116H mutant by Vanhove *et al.* (16) but must be confirmed by NMR and perturbed angular correlation experiments and by resolution of the three-dimensional structure of the N116H and N116H/N220G mutants. Unfortunately, no suitable crystals have been obtained yet.

With both mutants, the zinc concentration did not modify the K_m values, but k_{cat} decreased for imipenem or increased for the other substrates. Such a "non-competitive" inhibition or activation suggests that the substrate does not modify the affinity for the second zinc, and *vice versa.* Accordingly, the K_D2 values measured on the basis of the activation or inhibition curves were similar within the limits of experimental errors: 86 and 110 μM for N220G, and 3-9 μM for N116H/N220G (Table VIII).

From a B2 to a B3 Metallo-β-lactamase

The Arg^{121} residue is conserved in the BcII and CphA enzymes. Dal Peraro *et al.* (23) have shown by quantum chemistry calculations that, in the mono-zinc form of BcII, Arg^{121} anchors the Asp^{120} side chain by forming a strong ionic bond, ultimately orienting the Zn(II)-bound hydroxide for nucleophilic attack of the antibiotic β-lactam ring. Moreover, Rasia *et al.* (24) have shown that the R121H mutation in BcII leads to poor positioning of Asp^{120}, and thus the k_{cat} values of this mutant are lower than those of WT BcII. Our kinetic results are consistent with a similar role for the Arg^{121} residue in CphA, because the k_{cat} value for imipenem is strongly decreased with R121H.

A similar conclusion can be reached by comparing the kinetic parameters of the triple mutant to those of N116H/N220G. The k_{cat} values are decreased as in the R121H mutant of BcII. However, the triple mutant, which is much less active than N116H/N220G, conserved a relatively broad substrate spectrum with significant hydrolysis rates of benzylpenicillin and cephalosporins. Moreover, it was the only mutant for which the binding of the second zinc *increased* the activity *versus* imipenem. Unfortunately, it was not possible to construct proteins more similar to the B3 enzymes, because the mutants with the additional C221S mutation could not be produced or were too unstable to allow detailed studies.

The mutants exhibit another characteristic of B3 enzymes, namely a strongly increased affinity for the second Zn^{2+} ion. Indeed, the K_D2 value decreased to below 1 μM. Thus, the replacement of the positive Arg^{121} side chain by a neutral one enhances the affinity for Zn^{2+}, as was also suggested for BcII (8, 25) and CcrA (9). Rasia and Vila (24) have shown that the R121H mutation increases the affinity for the second Zn^{2+} ion in BcII and that the H121 side chain replaces His^{263} as a ligand of the second Zn^{2+}.

In contrast, the R121C mutation does not increase the affinity for the second Zn^{2+} in BcII (26), and the C121R mutant of CcrA was isolated in the di-zinc form, although the removal of the second zinc appeared to be facilitated (27). All these data and our results indicate that the replacement of Arg^{121} by a neutral residue in CcrA, IMP-1, and the sub-class B3 enzymes increases the affinity for the second zinc. Indeed, no mono-Zn^{2+} form of sub-class B3 enzymes has ever been obtained.

CONCLUSION

Starting with the narrow spectrum *A. hydrophila* B2 β-lactamase, we have obtained, after introducing two mutations, an enzyme with a much broader activity spectrum. The ratio between the k_{cat}/K_m for different antibiotics and imipenem calculated for different class B β-lactamases and the CphA mutants underscores the complete modification of the CphA activity. As the subclass B1and B3 enzymes, the N116H/N220G mutant is able to hydrolyze with a similar efficiency carbapenems, penicillins, and cephalosporins (Table X).

Moreover, rather than being inhibited by a second zinc, the double mutant is activated to various degrees (depending upon the substrate) by the addition of a second zinc with a K_D2 of about 5 µM. The only exception is imipenem, for which a 50% decrease of activity is observed, but with the same K_D2 value. It remains to be determined if, in the mononuclear double mutant, the sole Zn^{2+} ion is distributed between the two sites as in BcII (28).

Although obtaining a mutant similar to sub-class B3 enzymes proved more difficult, the mutants containing the R121H substitution exhibited an increased affinity for the second zinc (a B3 characteristic), and the triple mutant, although less active than the double one, also exhibited a broadened substrate profile. The double mutant N116H/N220G represents an ideal starting point to test the ability of the enzyme to adapt to the hydrolysis of various substrates or groups of substrates by directed evolution.

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