Kinetic and spectroscopic characterization of native and metal-substituted β-lactamase from *Aeromonas hydrophila* AE036

Maria Hernandez Valladares[a,b], Martin Kiefer[a], Uwe Heinz[a], Raquel Paul Soto[a,b], Wolfram Meyer-Klaucke[c], Hans Friederich Nolting[c], Michael Zeppezauer[a], Moreno Galleni[b], Jean-Marie Frère[b], Gian Maria Rossolini[d], Gianfranco Amicosante[e], Hans-Werner Adolph[a,*]

[a] *Fachrichtung 12.4 Biochemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany*
[b] *Centre d'Ingenierie des Proteines (B6), Université de Liège, Sart Tilman, 4000 Liège, Belgium*
[c] *EMBL-Outstation Hamburg at DESY, Notkestr. 85, D-22603 Hamburg, Germany*
[d] *Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Universita di Siena, 53100 Siena, Italy*
[e] *Dipartimento di Scienze e Tecnologie Biomediche e di Biometria, Universita dell'Aquila, 67100 L'Aquila, Italy*

Received 11 December 1999

Edited by Barry Halliwell

Abstract Two metal ion binding sites are conserved in metallo-β-lactamase from *Aeromonas hydrophila*. The ligands of a first zinc ion bound with picomolar dissociation constant were identified by EXAFS spectroscopy as one Cys, two His and one additional N/O donor. Sulfur-to-metal charge transfer bands are observed for all mono- and di-metal species substituted with Cu(II) or Co(II) due to ligation of the single conserved cysteine residue. Binding of a second metal ion results in non-competitive inhibition which might be explained by an alternative kinetic mechanism. A possible partition of metal ions between the two binding sites is discussed.

Key words: Metallo-β-lactamase; Metal exchange; EXAFS; UV-vis spectroscopy; Kinetics

1. Introduction

β-Lactamases are extracellular or periplasmic enzymes synthesized by bacteria. The producing strains become resistant to β-lactam antibiotics. Class B β-lactamases [1,2] are metalloproteins which require bivalent transition-metal ions for activity, and Zn^{2+} appears to be the metal present in the naturally occurring proteins.

The number of known class B β-lactamase producers has significantly increased during the last decade. However, only a few enzymes of this group have been studied in detail. The three-dimensional (3D) structures of the enzymes from *Bacillus cereus* 569/H/9, *Bacteroides fragilis* CcrA and *Stenotrophomonas maltophilia* have been solved, revealing the presence of one [3] or two zinc ions [4] in the active site of the *B. cereus* enzyme and of two zinc ions in those of the *B. fragilis* [5] and *S. maltophilia* enzymes [6]. The ligands of the single zinc ion in the X-ray structure of the *B. cereus* enzyme are three histidines and one water molecule in the so-called first binding site. By UV-vis difference spectroscopy of the Co_1^{2+}-*B. cereus* enzyme [7], however, a cysteine residue was also identified as metal ligand. The ligands for one of the zinc ions bound in the active site of the *B. fragilis* and *S. maltophilia* enzymes are also three histidines, whereas the second is coordinated by one aspartate, one cysteine, one histidine and one water molecule in the *B. cereus* and *B. fragilis* enzymes, and by two histidines, one aspartate and two water molecules in the *S. maltophilia* enzyme. The *Aeromonas hydrophila* enzyme retains the same set of metal ligands as the *B. cereus* and *B. fragilis* enzymes except that His-86 in the first binding site is replaced by an asparagine residue (the numbering is that of the *B. cereus* enzyme).

The catalytic activity of the *B. fragilis* enzyme versus nitrocefin seemed to be correlated with the simultaneous occupancy of both Zn binding sites [8]. Recently it could be shown that the enzyme is also active as the Zn_1-enzyme with even increased activity versus cefoxitin as the mononuclear enzyme [9]. For the enzyme from *B. cereus* it could be shown that mono- and binuclear species are active with unchanged or slightly increased turnover numbers for the binuclear enzyme, depending on the enzyme strain and the substrate used [10]. Conversely, the presence of a zinc ion in the second binding site of the *A. hydrophila* enzyme non-competitively inhibits its activity on imipenem [11].

The enzyme from *A. hydrophila* differs from all other known class B β-lactamases with respect to its narrow substrate spectrum. In an attempt to identify the metal ion ligands we performed EXAFS spectroscopy of the native enzyme, and electronic spectroscopy of stoichiometrically defined metal ion-substituted derivatives. The spectroscopic results are correlated to metal ion affinities and catalytic properties of the different metal-substituted species.

2. Materials and methods

2.1. Preparation of enzyme species

The preparation of the native and metal-free (apo-) β-lactamase from *A. hydrophila* AE036 was described before [11,12]. The enzyme concentration was determined spectrophotometrically using $\varepsilon_{280} = 38\,500\ M^{-1}\ cm^{-1}$ for the Zn- and apoenzymes. The Co^{2+}- and Cd^{2+}-substituted forms of the enzyme were prepared by direct addition of metal ions to the apoenzyme in either metal-free 15 mM sodium cacodylate, pH 6.5, or 50 mM HEPES, pH 7.5. The Cu^{2+}-species were produced in 50 mM TES, pH 7.5. The Cu^{2+}-Zn^{2+}-hybrid was obtained by addition of one equivalent of Zn^{2+} to the Cu_2^{2+}-enzyme at pH 7.5.

*Corresponding author. Fax: (49)-681-3022097.
E-mail: hwadolph@rz.uni-sb.de

Abbreviations: EXAFS, extended X-ray absorption fine structure; LMCT, ligand-to-metal charge transfer

PII: S0014-5793(00)01102-9

2.2. Metal ion binding constants, UV-vis spectroscopy, kinetics

The dissociation constant for a first metal ion bound (K_D') was obtained from competition experiments with the chromophoric chelator quin-2 (Molecular Probes, Eugene, OR, USA) for Zn^{2+} and Cd^{2+} [13], and with phen-green (Molecular Probes, Eugene, OR, USA) for Cu^{2+}. The indicators were titrated with metal ions in the absence and presence of the apoenzyme (protein/indicator ratios were 1:1, 1:2 and 1:3). Changes in absorbance at 266 (quin-2) and at 493 nm (phen-green) were monitored. The program Chemsim was used for numerical data analysis [14].

The dissociation constants for a second Zn^{2+}, Cd^{2+}, Cu^{2+} or Co^{2+} ion bound (K_D'') were determined by equilibrium dialysis experiments at 4°C. Metal analysis was done by atomic absorption spectrometry using a Perkin Elmer 2100 atomic absorption spectrophotometer in the flame mode for Zn^{2+}, Cd^{2+} and Cu^{2+} or a Perkin Elmer 1100 atomic absorption spectrophotometer in the graphite furnace mode equipped with a HGA-400 Programmer for Co^{2+}.

The hydrolysis of imipenem was monitored by following the absorbance variations resulting from the cleavage of the β-lactam ring using $\Delta\varepsilon$ of 9000 M^{-1} cm^{-1} at 300 nm. Imipenem was a kind gift of Merck Sharp and Dohme, nitrocefin was purchased from Unipath (Oxford, UK). The K_m and k_{cat} values were derived from complete hydrolysis time courses [15] or initial rate measurements.

The UV-vis spectra of the various enzyme species (40–50 μM) were recorded at 25°C with a Lambda 2 spectrophotometer from Perkin-Elmer.

2.3. Extented X-ray absorption fine structure (EXAFS) spectroscopy of the Zn^{2+}-enzyme

The mono-Zn^{2+}-enzyme samples were prepared by successive dialysis steps of the enzyme against 10 mM Bis–Tris buffer, pH 6.5 and 10 mM Tricine buffer, pH 7.5. After centrifugation, the samples were freeze-dried. The solutions were prepared from freeze-dried enzyme and the final concentration adjusted to 1.5 mM.

The K-edge EXAFS data were collected at beam line D2 (European Molecular Biology Laboratory Outstation Hamburg, Germany) with a positron beam energy of 4.6 GeV and a maximum stored current of 100 mÅ in fluorescence mode [16]. The measurements were performed with either frozen solutions or freeze-dried powders at 18K. The energy resolution was better than 2.5 eV. The data were analyzed using the computer program packages EXPROG (developed by C. Hermes and H.F. Nolting at the EMBL-Outstation) and EXCURV92 (developed by N. Binsted, S.W. Cambell, S.J. Gurman and P. Stepherson at SERC Daresbury). For the EXAFS data analysis and the refinement of the structural parameters, restraints were included as described by Binsted et al. [17].

3. Results

3.1. Metal ion affinities and kinetic parameters of native and metal-substituted enzyme species

Table 1 compares the dissociation constants for a first (K_D') and a and second metal ion bound (K_D'').

The affinity for a first metal ion decreases in the order $Zn^{2+} > Cd^{2+} > Co^{2+} > Cu^{2+}$ with little variation over the pH range 6.5–7.5, whereas the dissociation constant of Zn^{2+} is considerably increased to 120 ± 14 nM in 50 mM Na–cacodylate, pH 5.5. The affinity for a second metal ion decreases in the order $Cu^{2+} > Zn^{2+} > Cd^{2+} > Co^{2+}$ at pH 6.5 and $Cd^{2+} > Zn^{2+} > Cu^{2+} > Co^{2+}$ at pH 7.5.

The influence of the catalytic metal ions on activity was studied with imipenem which is the most efficiently hydrolyzed substrate of the *A. hydrophila* Zn-β-lactamase [18]. Kinetic data for the native and metal-substituted species are combined in Table 2. Binding of a second Cd^{2+} to the mono-cadmium enzyme at pH 6.5 results in non-competitive inhibition, as it has been previously described for the zinc enzyme [11]. The k_{cat} and K_m values of the Cu_2^{2+}- and Co_2^{2+}-enzymes could not be determined since the necessary excess of both metal ions leads to formation of complexes with β-lactams [19,20].

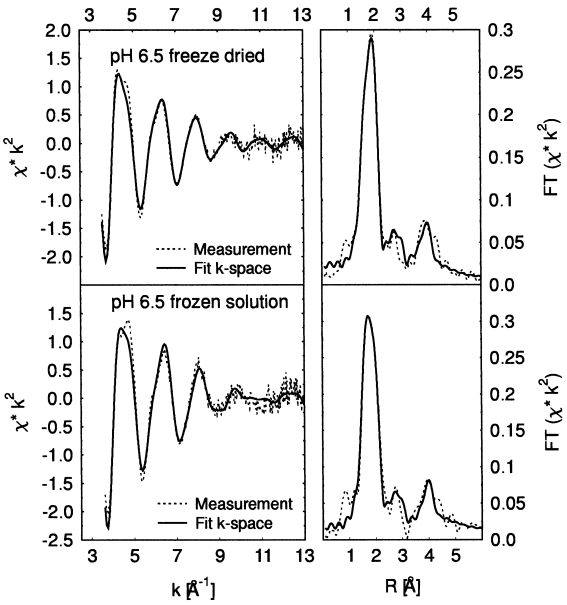

Fig. 1. EXAFS spectra of Zn_1^{2+}-β-lactamase from *A. hydrophila* at pH 6.5. The EXAFS spectra of a freeze-dried sample (top) and of a frozen solution (bottom) with theoretical fits are shown to the left and the corresponding Fourier transforms to the right. The fits were obtained with a coordination environment composed of 1 sulfur from cysteine, 2 nitrogens from histidine and 1 additional N/O donor (compare [21]).

3.2. EXAFS spectroscopy of the Zn_1^{2+}-enzyme

To avoid interference of heavy atoms in buffers such as sulfate or cacodylate with EXAFS measurements, Bis–Tris and Tricine were used at pH 6.5 and 7.5, respectively. The dissociation constants obtained for a single Zn^{2+} bound were 2 ± 1 and 8 ± 2 pM in Bis–Tris and Tricine buffers, respectively, and the k_{cat}/K_m values 2.7 and 2.5×10^6 M^{-1} s^{-1}. The frozen solution and the freeze-dried samples retained 85 and 90% of activity after irradiation, respectively.

The rigid structure of systems like imidazole is well known. Therefore in EXAFS restrained refinement is applied to such problems [17]. This allows the bond length and angles within the imidazole ligand to change slightly. The Debye-Waller parameters were assumed to be identical if the distances between central Zn-atom and backscattering atoms were similar. This considerably reduces the number of free parameters. The amplitude reduction factor accounting for inelastic losses was fixed to 0.925. The interpretation of the extracted k^2-weighted fine structure by these assumptions results in the two fits shown in Fig. 1 at pH 6.5. Data of a freeze-dried sample at pH 7.5 indicated the same geometry (data not shown). The Fourier transform in Fig. 1 clearly shows that the assumed coordination of two histidines matches the typical imidazole pattern at about 2.6 and 4 Å. The peak positions in the Fourier transform differ from the geometric distances due to the phase correction one accounts for in the refinement.

The first peak in the Fourier transforms reflects nitrogen atoms of imidazole, oxygen of a N/O donor ligand or OH^-, and sulfur from a cysteine ligand bound to the zinc. Such a ligand composition results in very good fits shown in Fig. 1. It

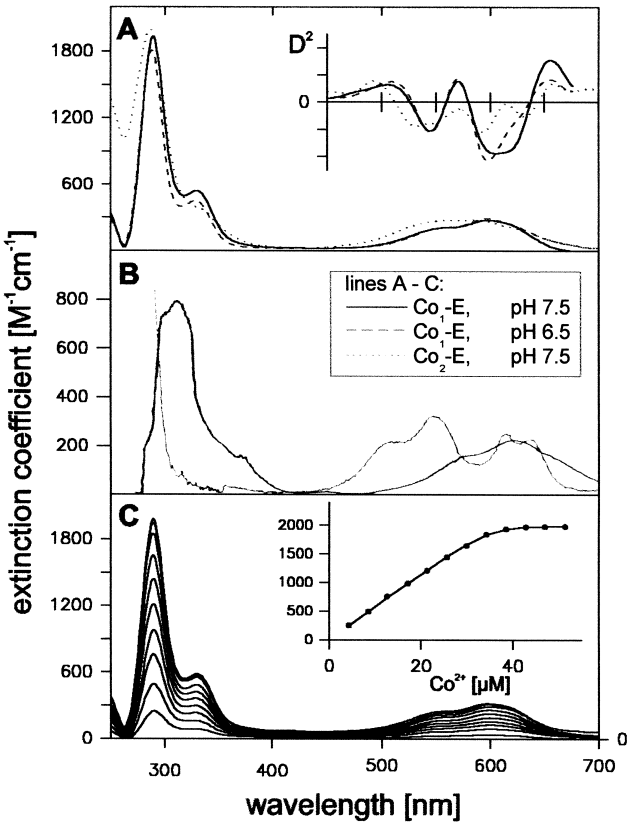

Fig. 2. UV-vis spectra of Co^{2+}-substituted β-lactamase from *A. hydrophila*. A: UV-vis difference spectra of Co_1^{2+}-β-lactamase at pH 6.5 and 7.5 and of the Co_2^{2+}-β-lactamase at pH 7.5. The spectra were recorded at 25°C. The second derivatives of the visible spectra are shown as inset. B: UV-vis difference spectra of Co(II)-substituted native carbonic anhydrase (thin line) and a His-94→Cys mutant (thick line). The figure has been obtained by scanning the published spectrum [24]. C: Titration of the LMCT and d-d transition bands upon addition of Co(II) ions to the apoenzyme (40 μM) at pH 7.5, 25°C. The titration curve of the apo-β-lactamase at 289 nm is shown as inset. The experimental data were fitted to a hyperbolic function.

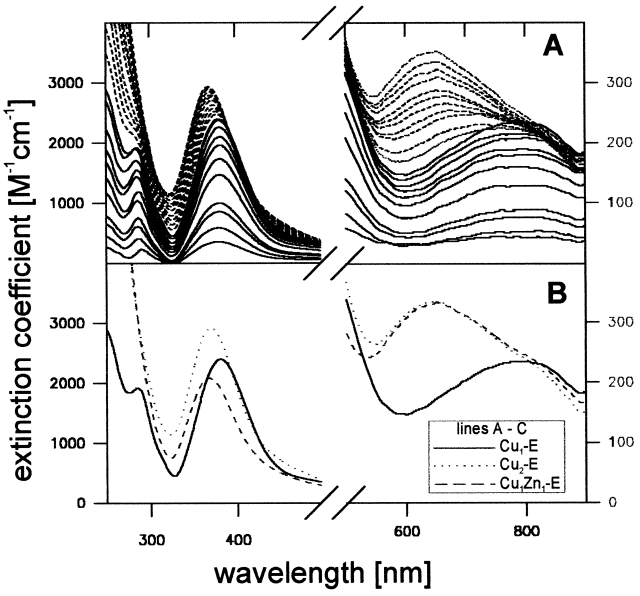

Fig. 3. UV-vis spectra of Cu^{2+}-substituted β-lactamase from *A. hydrophila*. A: Titration of the apoenzyme (50 μM) with Cu(II) ions as visualized by LMCT and d-d transition bands at pH 7.5, 25°C up to a second equivalent bound. B: UV-vis difference spectra of the Cu_1^{2+}-enzyme, Cu_2^{2+}-enzyme and Zn^{2+}-Cu^{2+}-hybrid enzyme, at pH 7.5 and 25°C. The spectrum of the hybrid form was recorded after addition of one equivalent of zinc ions to the Cu_2^{2+}-enzyme.

seems evident that a coordination number of 1 for cysteine sulfur as a ligand to the single zinc ion has to be taken into account.

3.3. Electronic spectroscopy of the Co^{2+}- and Cu^{2+}-substituted enzyme

The UV-vis difference spectra of the Co_1^{2+}-enzyme are characterized by two intense sulfur-to-Co^{2+} ligand-to-metal charge transfer (LMCT) bands in the UV region ($\varepsilon_{289} = 1800$ M^{-1} cm^{-1} and 1940 M^{-1} cm^{-1}, $\varepsilon_{325} = 450$ M^{-1} cm^{-1} and 530 M^{-1} cm^{-1} at pH 6.5 and 7.5, respectively) and additional bands between 500 and 650 nm ($\varepsilon_{600} = 275$ M^{-1} cm^{-1} and 290 M^{-1} cm^{-1} at pH 6.5 and 7.5, respectively) most likely due to Co^{2+} d-d transitions (Fig. 2A). The positions of both UV bands are unchanged in the Co_2^{2+}-enzyme at pH 7.5. The absorption changes between Co_1^{2+}- and Co_2^{2+}-species in the visible range (compare second derivatives in Fig. 2A) are characterized by an unchanged ε at 600 nm and an increase by 75 M^{-1} cm^{-1} at 550–560 nm. The intensity of either the LMCT or d-d bands displayed by the enzyme upon titration with Co^{2+} (Fig. 2C) at pH 6.5 and 7.5 could be used to determine the K_D' values shown in Table 1.

The UV-vis spectrum of the Cu_1^{2+}-enzyme at pH 7.5 exhibits absorption maxima at 290 nm ($\varepsilon = 1950$ M^{-1} cm^{-1}), 381 nm ($\varepsilon = 2300$ M^{-1} cm^{-1}) and a shoulder at 795 nm ($\varepsilon = 220$ M^{-1} cm^{-1}) (Fig. 3A). For the Cu_2^{2+}-species the 381 nm band is shifted to 369 nm ($\varepsilon = 2890$ M^{-1} cm^{-1}) and an additional absorption band is observed at 655 nm ($\varepsilon = 370$ M^{-1} cm^{-1}).

Table 1
Dissociation constants for a first (K_D') and a second (K_D'') metal ion bound to the various forms of the *A. hydrophila* metallo-β-lactamase

Sample	pH 6.5		pH 7.5	
	K_D'	K_D''	K_D'	K_D''
Zn^{2+}-enzyme	7 ± 2 pM[a]	40 ± 6 μM[c]	6 ± 2 pM[a]	10 ± 3 μM[c]
Cd^{2+}-enzyme	60 ± 10 pM[a]	82 ± 8 μM[c]	80 ± 7 pM[a]	4 ± 2 μM[c]
Co^{2+}-enzyme	220 ± 25 nM[d]	> 5 mM[c]	330 ± 40 nM[d]	500 ± 48 μM[c]
Cu^{2+}-enzyme	620 ± 55 nM[b]	< 10 μM[c]	550 ± 58 nM[b]	< 20 μM[c]

The K_D values are averages of four determinations.
[a]Data obtained using quin-2 as chelator.
[b]Data obtained using phen-green as chelator.
[c]Data obtained from equilibrium dialysis experiments.
[d]Data obtained from titration of the charge transfer and d-d transition bands.

Table 2
Kinetic parameters for hydrolysis of imipenem by the mono- and di-substituted *A. hydrophila* β-lactamase

Enzyme (E)	pH 6.5				pH 7.5			
	$[M^{2+}]$ (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	$[M^{2+}]$ (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
Zn-E	0.4	1300	180 ± 20	7.2	0.4	625	100 ± 11	6.3
Zn_2-E	200	240	170 ± 25	2.0	100	–	–	0.25
Cd-E	0.5	20	15 ± 3	1.3	0.5	–	–	0.7
Cd_2-E	800	3	13 ± 3	2.3	50	–	–	0.1
Co-E	3	210	100 ± 8	2.1	3	80	90 ± 8	0.9
Cu-E	6	30	35 ± 5	8.6	6	–	–	0.9

Imipenem was added after previous incubation of the enzyme with the corresponding metal ion concentrations. The maximal activities were reached after 60 min at 25°C. Unless otherwise stated, S.D. values were below 10% of the mean of four different measurements. In some cases, only the k_{cat}/K_m values were determined from first-order time courses at low substrate concentrations. The enzyme concentrations were 2 and 8 nM for the Zn^{2+}- and Co^{2+}-substituted enzymes, respectively, and 200 nM for the Cd^{2+}- and Cu^{2+}-enzymes.

The bands at 290 nm and in the 365–385 nm range can be attributed to sulfur-to-copper LMCT transitions. The strong absorption maximum at $\lambda < 300$ in the spectrum is probably due to imidazole-to-copper LMCT transition(s) [22]. The bands in the visible region of the spectra of the Cu_1^{2+}- and Cu_2^{2+}-species can be attributed to d-d transitions due to Cu^{2+} in distorted four-coordinate sites [23]. The positions of the d-d band and the LMCT band at 368 nm are identical in the Cu^{2+}-Zn^{2+}-hybrid and in the Cu_2^{2+}-species with unchanged intensity of the d-d band and a decrease of ε_{368} to 2100 M^{-1} cm^{-1} in the hybrid (Fig. 3B).

4. Discussion

The only direct structural information so far available for the coordination environment of the active site zinc ion of the *A. hydrophila* Zn-β-lactamase results from the presented EXAFS investigation. Spectra obtained for the Zn_1-enzyme (Fig. 1) could be fitted with a tetracoordinated ligand geometry composed of 1 sulfur from Cys-168 at 2.27 Å, 2 nitrogens from histidines and 1 oxygen/nitrogen ligand at 2.00 Å. The possibility to fit the EXAFS spectra with one cysteine ligand at full occupancy is at variance with results obtained for the Zn_1^{2+}-species of the *B. cereus* enzyme, strain 5/B/6, where a fractional contribution of sulfur to the first coordination sphere gave the best fitting results [10,21]. From the available crystal structures of the *B. cereus* and *B. fragilis* enzymes [3,5] it becomes obvious that the conserved Cys residue cannot act as a metal ligand in the first or high affinity site of these enzymes. Thus it could be concluded that the Zn(II) ion in the *B. cereus* Zn_1-enzyme was distributed between the two binding sites with possible implications for the catalytic mechanism [10,21]. Although the enzymes from *A. hydrophila* and *B. cereus* show 35% of identical residues, minor changes in the tertiary structure could lead to a possible structural modification of the metal ion binding sites.

The UV-vis spectrum of the Co_1^{2+}-enzyme clearly exhibits sulfur-to-Co^{2+} LMCT bands at 289 and 325 nm and furthermore shows close resemblance with that of a Co^{2+}-substituted His-94-Cys mutant of carbonic anhydrase [24] (compare Fig. 2B), where 2 His and 1 Cys are the protein ligands in a distorted tetrahedral geometry. It can be concluded that Co^{2+} in the mono-metal species is also bound to cysteine as it has been shown for Zn^{2+} by EXAFS spectroscopy.

The minor spectral variations observed upon binding of a second Co^{2+} to Co_1^{2+}-β-lactamase from *A. hydrophila* are paralleled by recently published spectra of the Co^{2+}-substituted enzyme from *B. fragilis*: a first Co^{2+} bound obviously causes already $> 80\%$ of the absorbance of the Co_2^{2+}-species in the charge transfer as well as the d-d region (compare Fig. 3 in [25]). The weak d-d transitions contributed by a second Co^{2+} bound to the enzyme from *A. hydrophila* could be the result of an increased coordination number as it was found crystallographically for Zn^{2+} in the second binding site of the *S. maltophila* enzyme [6].

The difference of dissociation constants for a first and a second Cu^{2+} bound is significantly smaller as compared to Zn^{2+} and Co^{2+} (Table 1) and a partition of Cu^{2+} between the two sites in the Cu_1^{2+}-enzyme and in the Zn^{2+}-Cu^{2+}-hybrid could explain the spectroscopic results. In all cases sulfur-to-copper LMCT bands are observed with the highest intensity for the Cu_2^{2+}-species (Fig. 3B). The identity of band positions and intensities in the d-d region of the absorption spectra are giving no evidence to decide whether Cu^{2+} in the Zn^{2+}-Cu^{2+}-hybrid enzyme is preferentially bound to a specific binding site.

Although Zn^{2+} preferentially binds to a ligand set including Cys in the Zn_1^{2+}-species the Zn^{2+}-Cu^{2+}-hybrid shows a strong contribution of sulfur-to-Cu^{2+} charge transfer. This seems surprising since the relative magnitude of affinities for Zn^{2+} and Cu^{2+} differs by five orders of magnitude for a first metal ion bound whereas binding of a second metal ion is comparably strong for both metal ions. The observation of LMCT bands for the hybrid implies that Zn^{2+} and Cu^{2+} compete for Cys sulfur or share it as a bridging ligand. The reduced intensity of the LMCT bands in the Zn_1^{2+}-Cu_1^{2+}-hybrid could be the result of a partial occupation of the Cys binding site by Cu^{2+}. Very similar results have been obtained with a Zn-Co-hybrid enzyme which could only be produced with large excess of Co^{2+} (2 mM) causing turbidity of enzyme solutions (data not shown).

The large deviation between K_D' and K_D'' for all the metal ions studied might be the result of strong negative cooperativity connected to binding of a second metal ion.

As it has been observed with other zinc enzymes, the Co_1^{2+}-enzyme is the most active one after the natural Zn_1^{2+}-species with imipenem as substrate (Table 2). Binding of a second Zn^{2+} non-competitively inhibits the enzyme in the 5.5–8.0 pH range. At pH 6.5, a similar effect was observed upon binding of a second Cd^{2+} to the Cd_1^{2+}-species. For the Cu^{2+}- and Co^{2+}-enzymes, the same experiments could not be performed because of the direct interactions between the free ions and carbapenems interfering with the activity measurements.

Although Zn-β-lactamase from *A. hydrophila* has two conserved metal ion binding sites it is surprising that the binuclear enzyme species are non-competitively inhibited by a second metal ion bound. Therefore it may be concluded that the mono-nuclear species represents the native enzyme. Comparable to the interpretations of experimental results derived for the enzyme from *B. fragilis* [9] and *B. cereus* [10] a role of a second metal ion binding site during catalysis may be suggested. A transient occupation of this site by zinc with a bound reaction intermediate could explain its presence. In case of the binuclear enzymes such a translocation would be superfluous. The non-competitive inhibition observed for the *A. hydrophila* enzyme could then be interpreted in terms of a less efficient catalytic mechanism of the binuclear enzyme.

Acknowledgements: Supported by the European research network on metallo-β-lactamases, within the TMR Program (FMRX98-0232) and by grants from the Belgian Government (PAI P4/03), from the Deutsche Forschungsgemeinschaft (Ad 152/1-1) and from the Bundesministerium für Bildung und Forschung, contract 05SN8TSA1. We thank Dr. Bernhard Wannemacher and Wolfgang Reinle for help in the atomic absorption spectroscopic measurements.

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