J. Synchrotron Rad. (1999). **6**, 400–402

A Comparison of *Bacillus Cereus* and *Aeromonas Hydrophilia* Zn-β-lactamases

Wolfram Meyer-Klaucke,[a]* Raquel Paul Soto,[bc] Maria Hernandez Valladares,[bc] Hans-Werner Adolph,[b] Hans-Friederich Nolting,[a] Jean-Marie Frère[c] and Michael Zeppezauer[b]

[a]European Molecular Biology Laboratory, Outstation Hamburg, Notkestr. 85, 22603 Hamburg, Germany, [b]Fachrichtung 12.4 Biochemie, Universität des Saarlandes, 66041 Saarbrücken, Germany, [c]Centre d'Ingéniérie de Protéines, Institut de Chimie B6, Université de Liège, Sart-Tilman, B-4000 Liège, Belgium. Email:Wolfram.Meyer-Klaucke@embl-hamburg.de

In an increasing number of pathogenic bacteria Zn-β-lactamases are being discovered as major factors of antibiotic resistance. For a possible strategy to fight these bacteria, it is important to understand the catalytic mechanism. X-ray absorption spectroscopy measurements on the zinc-K-edge of β-lactamases from *Bacillus cereus* (strain 5/B/6) and *Aeromonas hydrophila* (AE 036) gives evidence for differences in the mode of zinc sulfur coordination and information about the coordination to N and O donor ligands.

Keywords: biological system, enzyme, beta-lactamase, EXAFS, zinc

1. Introduction

Zn-β-lactamases catalyze the hydrolysis of penicillin and cephalosporin antibiotics by cleavage of their β-lactam ring. The production of Zn-β-lactamases most often renders bacteria resistant to almost all β-lactam drugs so far designed.

The number of Zn-β-lactamase producing bacteria has notably increased in this decade. However, only few enzymes of this group have been studied in detail. The 3D structure of the *Bacillus cereus* 569/H/9 enzyme at pH 5.7 revealed the presence of a zinc ion at the active site coordinated to 3 His residues (86, 88 and 149) and a water molecule [Carfi et al. (1995)]. This is in disagreement with UV-Vis spectroscopic studies on the monoCo-*B. cereus* [Myers & Shaw (1989); Orellano et al. (1998)] and monoCu-*B. cereus* derivatives [Hilliard (1995)] which suggest cysteine ligation. In a second structure the conserved Cys168 residue is coordinating together with His210 and Asp90 a second zinc ion, located 3.7 Å from the first. [Carfi et al. (1998)]. The set of ligands coordinating the two zinc ions is highly conserved in all the enzymes of the family. One exception is the *Aeromonas hydrophila* enzyme where His86 is replaced by an asparagine residue.

The *B. cereus* enzymes from 5/B/6 and 569/H/9 strains are active with only one zinc ion but binding of a second one may improve the catalytic efficiency in some cases [Paul Soto et al. (1998)]. This is in contrast to the *A. hydrophila* enzyme where binding of a second zinc ion has an inhibitory effect [Hernandez Valladares et al. (1997)].

We have performed EXAFS studies on the *B. cereus* 5/B/6 and *A. hydrophila* enzymes, both at pH 6.5 containing about 1 equivalent of Zn. Note that the *B. cereus* enzymes from strains 5/B/6 and 568/H/9 differ in 17 aminoacid substitutions; none of which is located at the active site. Therefore, although the crystal structure of the 5/B/6 enzyme is not known similar metal coordination patterns are expected. For the *A. hydrophila* enzyme no crystal structure is available at present.

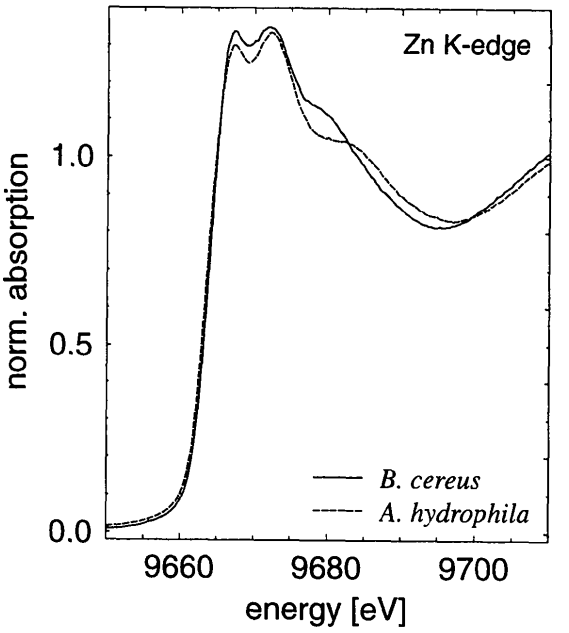

Figure 1
Normalized X-ray absorption edge for B. cereus (full line) and A. hydrophila (broken line). The variation of the intensities and positions of the resonances (e.g. at 9675 eV) suggest different zinc coordination for both enzymes.

2. Experimental section

The K-edge XAFS data were collected at beam line D2 at the European Molecular Biology Laboratory Outstation Hamburg with a positron beam energy of 4.6 GeV and a maximum stored current of 100 mA in fluorescence mode [Meyer-Klaucke et al. (1996)]. The samples were measured as frozen solutions at 18 K. An energy resolution of better than 2.5 eV has been achieved. The data have been analyzed using the computer program packages EXPROG [Nolting & Hermes (1992)] and EXCURV92 [Binsted et al. (1992)].

The rigid structure of systems like imidazole is well known. Therefore in EXAFS data analysis restrained refinement is applied to such problems [Binsted et al. (1992)]. This allows varying the bond length and angles within the imidazole ligand only slightly remaining the gross structure, whereas distance and angle between the Zn atom and the imidazole ring can be optimized. The Debye-Waller parameters were assumed to be identical if the distances between central Zn-atom and backscattering atoms are similar. This approach reduces considerably the number of free parameters. The amplitude

reduction factor accounting for inelastic losses was fixed to 0.925. The number of independent points (N_{idp}) is estimated to 24 using $\Delta k = 10$ and $\Delta R \geq 3.5$ ($N_{idp} = (2\,\Delta k\,\Delta R)/\pi + 2$).

3. Results and discussion

3.1 *Bacillus cereus 5/B/6*

From crystal structure data a coordination of the Zn atom by 3 His residues and one H_2O molecule would be expected. The interpretation of the extracted k^2 weighted fine structure by this assumption results in fit 1 shown in figure 2. The corresponding Fourier transform (same figure) clearly indicates a missing contribution at about 2.27 Å. We account this contribution to a cysteine ligand. From its amplitude it is obvious that in average less than one sulfur atom is present. To obtain an upper limit for the number of sulfur ligands the corresponding Debye-Waller parameter were fixed, because of their strong correlation with the coordination numbers in the EXAFS theory. The Debye-Waller parameter of the sulfur atom accounts only for dynamic disorder and the static disorder between all the enzyme units, whereas the Debye-Waller parameter for the nitrogen also bound to the central Zn additional accounts for the static disorder within this unit (between the 3 imidazole ligands). Thus the Debye-Waller parameter of the sulfur is expected to be much smaller than the one of the nitrogen. For our upper limit of sulfur atoms we fix it to a even slightly lower value (0.003 $Å^2$). Re-analyzing of the data with this model leads to a maximum sulfur coordination of 0.54 (5). This is shown in figure 2 as fit 2. The corresponding parameters, given in table 1, show that the improvement of the fit is only due to this sulfur contribution. Noticeable none of the other parameters has changed. Neglecting the restrains for the imidazole ligand a statistical F-test was performed in order to check the significance of fit 2 in respect to fit 1. This test results in a higher probability for fit No.2 (65 %). Using the restrained refinement the number of free parameters was even smaller, thus the probability of fit No.2 is higher than 65%.
The difference between the experimental and theoretical Fourier transforms clearly indicates that no further contribution is missing. All differences are only of the order of the noise level. Furthermore, no Zn-Zn distance can be significantly found, excluding the possibility of two zinc ions present in the same enzyme molecule. A quite similar zinc coordination for *B. cereus* has been reported earlier by Feiters [Feiters (1990)].

3.2 *Aeromonas hydrophila*

The Zn XANES of *B. cereus* and *A. hydrophila* shown in figure 1 differs for both enzymes. This variation is due to a different zinc environment, as expected from the enzyme sequences.

The contribution accounting for the imidazole ligands in the extracted k^2-weighted fine structure of *A. hydrophila,* shown in Figure 2, is less pronounced compared to *B. cereus.* This is consistent with the replacement of His86 by an aspargine residue. The first peak in the Fourier transforms at about 1.9 Å reflects the two nitrogen atoms of the imidazole, the oxygen atom of a N/O-donor ligand or OH, and the sulfur atom of the cysteine ligand bound to the Zn atom. The refined parameters are given in Table 1. From the sequence it is well known that at most one sulfur can bind to this metal atom. The analysis of the contour plot of these coordination numbers shows that for sulfur a lower coordination number than one and for imidazole a higher coordination number than two can be excluded.

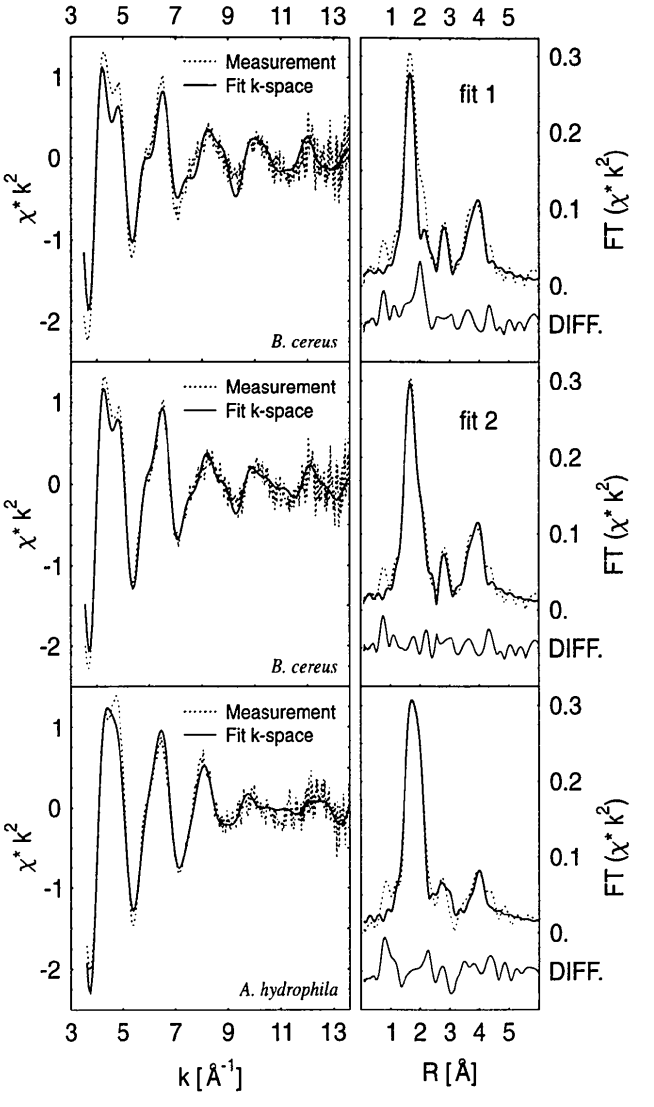

Figure 2
EXAFS results for the β-lactamases under study. The two models used for B. cereus are called fit 1 and fit 2. Below the Fourier transforms the difference between theory and measurement is plotted.

4. Conclusions

For *B. cereus* we have found at most 0.5 sulfur ligands. The X-ray structure however showed the sole Cys residue (Cys168) at a distance of 4.4 Å from the first zinc ion, too long for metal ligation. Thus we explain the Cys contribution to the EXAFS spectrum by a partial occupancy of the second binding site. In this case, the EXAFS spectrum represents an average of two different coordination geometries simultaneously present indicating that in the monoZn-species the zinc ion is dislocalized between the two binding sites. However our data do not allow to precise the occupancy of each binding site.

Ligand	*Bacillus cereus* fit 2 N_I	R_i[Å]	$2\sigma^2$ [Å^2]	*Bacillus cereus* fit 1 N_I	R_i[Å]	$2\sigma^2$ [Å^2]	*Aeromonas hydrophila* N_I	R_I[Å]	$2\sigma^2$ [Å^2]
His	3.0			3.0			2.0		
N		1.98(1)	0.004(1)		1.99(1)	0.004(1)		2.00(1)[c]	0.005(1)[d]
C		2.99(1)	0.015(2)[a]		2.99(1)	0.014(4)[a]		2.95(1)	0.007(2)[a]
C		3.05(1)	0.015(2)[a]		3.07(1)	0.014(4)[a]		3.12(1)	0.007(2)[a]
N		4.13(1)	0.008(2)[b]		4.14(2)	0.008(2)[b]		4.12(2)	0.008(3)[b]
C		4.19(1)	0.008(2)[b]		4.21(1)	0.008(2)[b]		4.23(1)	0.008(3)[b]
H_2O or O/N-donor	1.0			1.0			1.0		
O		3.27(4)	0.015(2)[a]		3.25(5)	0.014(4)[a]		2.00(1)[c]	0.005(1)[d]
Cys	0.54(5)			0.0			1.0		
S		2.27(1)	0.003		--	--		2.27(1)	0.003(1)
Fit index Φ		2.0			2.9			1.8	

Table 1

EXAFS results for B. cereus and A. hydrophila. All parameters with given error margins were adjusted by the refinement. Constrains are indicated by small letters a, b, c and d. Furthermore constrained refinement has been applied to the histidine ligands. Its coordination numbers attribute for all atoms of the imidazole ring. The possible H_2O or O/N-donor in B. cereus at about 3.25 Å was included to indicate that the authors favor a total coordination number of 4. Its backscattering signal does not separate from the first imidazole multiple-scattering peak very well. The energy-offset parameter differs for the fits slightly. (B. cereus: fit 1: -7.0(5)eV; fit 2: -6.0(3)eV; A. hydrophila: -5.8(4)eV).

The exclusive location of the zinc ion at the 3 His site in the 3D structure of the monoZn-species [Carfi et al. (1995)] could be peculiar of the low pH used to grow the crystals. It has been proposed that a translocation of the zinc ion between the two metal sites could be essential for catalysis [Paul Soto et al. (1998)].

For the *A. hydrophila* enzyme EXAFS provides the only structural information available so far for the coordination geometry at the active site. The coordination numbers derived from the data analysis suggest the presence of only one ligand geometry. In contrast to the *B. cereus* enzyme the Cys residue appears to coordinate at the so-called first binding site. The coordination geometry (2 His, 1 Cys and 1 O/N) could be obtained with His88 and His149 from the first metal site and Cys168 as additional ligand. The oxygen/nitrogen ligand donor observed could either be hydroxide/water or Asn-86, which replaces His in the sequence of other known Zn-β-lactamases [Hernandez Valladares et al. (1998)]. To our knowledge Asn has never been observed as a zinc ligand in native proteins and it proved to be a poor zinc ligand in proteins obtained by site-directed mutagenesis [Ma et al. (1995)].

This work was supported by the European research network on metallo-β-lactamases, within the Human Capital and Mobility Program (Contract N° ERBFMRX-CT98-0232) and by the Bundesministerium für Bildung und Forschung under contract 648FLA (Verbund 48). H.F.N. was not available for reading the manuscript.

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(Received 10 August 1998; accepted 16 December 1998)