Coordination geometries of metal ions in D- or L-Captopril-inhibited metallo-beta-lactamases

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Summary:

D- and L-captopril are competitive inhibitors of metallo-beta-lactamases. For the enzymes from *Bacillus cereus* (BcII) and *Aeromonas hydrophila* (CphA) we found that the mono-nuclear enzymes are the favoured targets for inhibition. By combining results from Extended X-ray Absorption Fine Structure (EXAFS), Perturbed Angular Correlation (PAC) of gamma-rays spectroscopy and a study of metal ion binding, we derived, that for Cd1-BcII, the thiolate sulfur of D-captopril binds to the metal ion located at the site defined by three histidine ligand residues. This is also the case for the inhibited Co1- and Co2-enzymes as observed by UV-Vis spectroscopy. Whereas the single metal ion in Cd1-BcII is distributed between both available binding sites in both the uninhibited and the inhibited enzyme, Cd1-CphA shows only one defined ligand geometry with the thiolate sulphur coordinating to the metal ion in the site composed of 1 Cys, 1 His and 1 Asp. CphA shows a strong preference for D-captopril which is also reflected in a very rigid structure of the complex as determined by PAC spectroscopy. For BcII and CphA, which are representatives of the metallo-beta-lactamase subclasses B1 and B2, we find two different inhibitor binding modes.
Introduction:

Metallo-beta-lactamases confer antibiotic resistance to bacteria by catalysing the hydrolysis of beta-lactam antibiotics, including carbapenems. This relatively new form of resistance is spreading and thereby escaping the effective inhibitors developed to fight the better known serine-β-lactamases. For all metallo-beta-lactamases investigated, structurally similar enzyme active sites comprising two zinc binding sites are reported. For BcII one metal-binding site contains three His (H-site); the other one 1 Asp, 1 Cys and 1 His as the metal ligating residues (DCH-site) as derived from X-ray crystallography (1). For CphA one His from the H-site (His 116) is supposed to be replaced by an Asn (2). Various thiol-carboxylate compounds were identified as potent inhibitors (3). The active site binding of thiomandelic acid to Bacillus cereus metallo-beta-lactamase (BcII) was studied by NMR spectroscopy (4) whereas the binding of 2-[5-(1-tetrazolylmethyl)thien-3-yl]-N-[2-(mercaptomethyl)-4-(phenylbutrylglycine)] to the enzyme from Pseudomonas aeruginosa (IMP-1) was characterized by X-ray crystallography (5). With both approaches the inhibited binuclear zinc enzymes were studied. Both studies agree in a bridging role of the metal-bound sulphur of the inhibitor while the carboxylate group of the inhibitors binds to an accessible amino acid, thus stabilizing the complex. Other known inhibitory compounds are 2,3-(S,S)-disubstituted succinic acids for IMP-1(6) or moxalactam and cefoxitin for CphA (7). The latter compounds lead to irreversible inactivation of the enzyme by the hydrolysed reaction products.

The structural investigation of D- and L-Captopril binding presented here is based on results obtained from enzyme kinetic and thermodynamic studies. Captopril is known as an Angiotensin converting enzyme (ACE)-blocking agent used in the therapy of blood pressure diseases.

Although different catalytic mechanisms for mono- and binuclear metallo-beta-lactamases have been discussed in the literature it is still not clearly understood why the enzymes have two conserved metal binding sites (for review see (8)). The motivation for the present investigation was the demand for a better knowledge of the nature of metal ion binding in presence of bound ligands. By a combination of Extended X-ray Absorption Fine Structure (EXAFS) and Perturbed Angular Correlation of γ-rays (PAC) spectroscopy we studied the nature of captopril interactions with the cadmium-substituted enzymes (detailed descriptions of the methods can be found in (9) and (10),
respectively). Both methods delivered consistent results which are additionally supported by UV-Vis spectroscopic results of Co(II)-substituted enzymes. The present investigation contributes new insights with respect to the physiological importance of mono- and binuclear metallo-beta-lactamases.

Materials and methods:
Production and characterization of enzymes and apo-enzymes

The metallo-beta-lactamases CphA from *A. hydrophila* AE036 and BcII from *B. cereus* 569/H/9 were purified as described (11;12). The protein concentrations were determined by measuring the absorbance at 280 nm using extinction coefficients of 30500 M⁻¹cm⁻¹ for BcII 569/H/9 and 38000 M⁻¹cm⁻¹ for CphA. Metal ion concentrations in samples and in the final dialysis buffers were determined by atomic absorption spectroscopy in the flame mode as described (13).

To produce “metal-free” buffers, buffer solutions in bi-distilled water were treated by extensive stirring with Chelex 100 (Sigma). Apo-enzymes were prepared by dialysis of the corresponding enzymes against 2 changes of 15 mM HEPES pH 7.0 containing 0.2 M NaCl and 20 mM EDTA over 12 h under stirring. EDTA was removed from the resulting apoenzyme solution by 3 dialysis steps against the same buffer containing 1 M NaCl and Chelex-100 and finally 2 dialysis steps against 15 mM HEPES pH 7.0 containing 0.2 M NaCl and Chelex-100. In all preparations the residual zinc content did not exceed 5 % as determined by atomic absorption spectroscopy.

Synthesis of D-Captopril

To synthesize D-Captopril (Scheme 1), we prepared compound 1 according to the procedure described in (14). Compound 2 was prepared following a method reported by Skiles et al. (15) and the classical hydrolysis reaction to obtain the D-captopril was carried out with NaOH 1N under an atmosphere of argon (16). As there are two asymmetric centres in the molecule, here D- and L-designations refer to absolute stereochemistry at the prolinyl stereocentre (see Figure 3).

Scheme 1

Determination of steady-state kinetic parameters and inhibition constants
All kinetic measurements were performed at 25°C with imipenem (kind gift of Merck Sharp and Dohme, Rahway, NJ, USA) as a substrate following the hydrolysis at 300 nm (Δε(300 nm) = -9000 M⁻¹cm⁻¹) in 15 mM HEPES, pH 7.0. The photometric measurements were either performed with a spectrophotometer (CphA) or the stopped-flow system DX-17MV (Applied Photophysics, Leatherhead, UK) in those cases where high concentrations of enzymes (0.1 - 1 µM) during the measurements were required to exactly define the reconstitution state of the metallo-enzymes. Under such conditions it was possible to study Zn₁- and Cd₁-BcII with apo-enzyme reconstituted with only 0.1 equivalents of metal without the interference of residual zinc in the metal-depleted buffers. The data evaluation was based on the concentration of metal ions added. Effects of residual zinc in the solutions could be minimized and it was possible to clearly discriminate Me₁- and Me₂-species. The steady-state parameters K_M and k_cat and the inhibition constants for D- and L-captopril were determined from initial rates. Standard non-linear regression analysis was used for data evaluation by directly fitting the Michaelis-Menten equation (un-inhibited or competitively inhibited) to the data. Activities of binuclear enzymes were studied with enzyme samples in presence of excess of the respective metal ions.

Inhibition constants for D- and L-Captopril were determined by variation of the inhibitor concentration at substrate concentrations fixed in the range of the respective K_M values.

**UV-Vis spectroscopy of Co(II)-substituted BcII**

UV-Vis spectra of Co(II)-substituted BcII were recorded with a Lambda9 spectrophotometer (Pekin-Elmer, Überlingen, Germany) and processed with the UV-Winlab software from Perkin-Elmer. Co₁-BcII was prepared by reconstitution of 130 µM apo-enzyme with 120 µM Co(II). Co₂-BcII was prepared by preincubation of 118 µM apo-BcII with 500 µM Co²⁺. To remove traces of precipitated protein the samples were centrifuged for 10 min at 30000 x g immediately before the measurement. The D-Captopril complexes were obtained by adding 800 µM D-Captopril to the sample cells.

**Determination of metal ion dissociation constants**

The dissociation constants for a first (K_{mono}) and second (K_{bis}) cadmium ion bound to BcII in presence of 25 µM D-Captopril and 0.1 M NaCl were obtained from competition experiments with the
chromophoric chelator Mag-fura-2 (MF1) (Molecular Probes, Eugene, Oregon) in 15 mM HEPES pH 7.0 as previously described (17;18).

X-ray absorption spectroscopy

XAS-sample preparation. The buffer used during purification has been exchanged for 20 mM Bis-Tris pH 7 by iterative use of Millipore’s Centricon devices to decrease scattering background. The final protein concentration was ~2-3 mM. The free metal concentration was below 2 µM. Samples have been frozen and stored at −20°C.

XAS-measurements. For x-ray absorption spectroscopy (XAS), about 100-120 µl of enzyme solution were transferred to the EXAFS cuvettes covered with Kapton tape (DuPont) as an X-ray transparent window material, capped, mounted on the sample holder, dropped into liquid nitrogen and transferred to the beamline cryostat. The Cd- K-edge (26711.0 eV) XAS was collected at the beamline D2 at DESY (EMBL outstation, Hamburg) running at 4.4 GeV and 70-125 mA current in fluorescence mode at 25 K sample temperature. An internal Cd-foil-sample was used for calibration.

XAS-Data analysis. Standard EXAFS analysis was performed using the EXPROG software package (developed by C. Hermes and H. F. Nolting at the EMBL-Outstation/Hamburg) to process the raw data and EXCURV98 (developed by N. Binsted, S. W. Cambell, S. J. Gurman and P. Stepherson at SERC Daresbury) using exact curve wave scattering theory (19;20) to analyse the spectra. The energy range was set to 30-650 eV above the edge. Phases were calculated \textit{ab initio} using Hedin-Lundqvist potentials and von Barth ground states (21). Both single and multiple scattering paths up to 4.5 Å from the metal atom were used to identify and quantify imidazole coordination of histidine ligands by using the program’s implemented small molecule database. After eliminating all non-imidazole atoms of the His unit, the complete imidazole ring was simulated by iterating the distance and Debye-Waller factor of the pivotal (directly coordinating) N atom and the angle phi of the second imidazole N atom for slight distance corrections of the constrained outer shell imidazole atoms. Debye-Waller factors of the outer shell atoms of imidazole rings were constrained assuming the Debye–Waller factors of atoms with similar distance to the absorber to be equal. Since the constraints
for the Debye-Waller factors are unique for each parameter set, details are summarized in Tables 2 and 3.

The fitting process included additional constraints for the following parameters. Two coordination clusters were introduced, each having an integer number of ligands. The fit then determined the fractional occupancy of each cluster for the mononuclear enzyme if the metal ions was distributed between the two metal sites. Theoretical simulations were generated by adding shells of scatterers around the central Cd atoms and iterating the number of scatterers, bond lengths and Debye-Waller factors in each shell. Additionally, the Fermi energy \( E_f \) (edge position relative to calculated vacuum position) was refined to achieve the best fit to the experimental data. The improvement of the fit after addition of each shell beyond the first was assessed by comparing the residual R-factor (22).

**Perturbed Angular Correlation of \( \gamma \)-rays spectroscopy**

\(^{111}\text{mCd}\) was produced by the Cyclotron Department at the University Hospital in Copenhagen, Denmark. Preparation and purification of \(^{111}\text{mCd}\) is described in (23). The PAC spectrometer is described in (24) and references therein.

In the case of identical, static and randomly oriented molecules, the perturbation function \( G_2(t) \) is

\[
G_2(t) = a_0 + a_1 \cos(\omega_1 t) + a_2 \cos(\omega_2 t) + a_3 \cos(\omega_3 t)
\]

with \( \omega_1, \omega_2 \) and \( \omega_3 \) as the three difference frequencies between the three sublevels of the spin 5/2 state of the cadmium nucleus (25). Note that \( \omega_1 + \omega_2 = \omega_3 \). Thus the Fourier transform of \( G_2(t) \) exhibits three frequencies for each nuclear quadrupole interaction (NQI). The Fourier transformation was performed as described in (24). The NQI is characterized by the numerically largest diagonal element after diagonalisation, chosen as \( \omega_{zz} \) which is denoted \( \omega_0 \) and \( \eta = (\omega_{xx} - \omega_{yy})/\omega_{zz} \). The relation between these two parameters and the frequencies in \( G_2(t) \) can be found in (23). Thus from the time dependence of \( G_2(t) \), \( \omega_0 \) and \( \eta \), determined through least squares fitting reflect the coordination geometry of the cadmium ion.

In the liquid state the NQI is time dependent because of the Brownian reorientation of the protein, described by the rotational diffusion time \( \tau_R \). This has the consequence that \( G_2(t) \) converges to
0 as a function of time, representing thermal equilibrium and isotropy in the angular correlation between the two gamma-rays.

The perturbation function $A^2G^2(t)$, where $A^2$ is the amplitude, was analysed by a conventional non-linear least squares fitting routine. Satisfactory fitting was obtained with a relative Gaussian distribution $\delta = \Delta\omega_0 / \omega_0$ applied to all the three frequencies. Non-zero values for $\delta$ indicate that the $^{111}$Cd nuclei are located in a distribution of surroundings. A NQI is then described by the parameters $\omega_0$, $\eta$, $\delta$ and $\tau_R$. In cases where more than a single NQI is present, the perturbation function is the sum of the different perturbation functions, where each NQI is weighted by its population (23).

**Results**

We have studied the interaction of metallo-beta-lactamases with the two diastereomers of Captopril, which proved to be competitive inhibitors of both BcII and CphA. Inhibition constants of D- and L-captopril for Me$_1$- and Me$_2$-BcII were determined with imipenem as the substrate (Table 1). Since it was not possible to yield reasonable results for the putative binuclear species of CphA only results for the mononuclear species are presented.

We have investigated the influence of inhibitor binding on metal dissociation constants. In presence of 25 $\mu$M of D-Captopril and 5$\mu$M BcII a first Cd ion binds with a $K_D$ of 1.6 nM compared to 8.3 nM in absence of D-Captopril (18). A second cadmium ion is bound with a $K_D$ of 50 $\mu$M compared to 5.9 $\mu$M in absence of the inhibitor (18). These $K_D$ values are macroscopic constants and in case of the mononuclear enzymes do not reflect any binding site assignment (see below).

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>For CphA, the substitution of cadmium for zinc results in a drastic decrease of $K_M$ and $k_{cat}$. Binding of Captopril is much stronger to the Cd-species than to the Zn-species with a strong preference for D-captopril (Table 1).</td>
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*UV-visible spectroscopy*

Binding of Co(II) to the metal-free enzyme at a [Co(II)]/[enzyme] stoichiometry of 0.8, results in the appearance of a ligand-to-metal charge transfer (LMCT) band at 344 nm and bands in the d-d
transition region (400 nm to 700 nm). In general the intensity of the LMCT bands is mainly due to Cys (sulphur)-Co interaction while the d-d transitions are caused by the His-Co interaction (17).

Figure 1

Increasing the [Co(II)]=[enzyme] ratio above 1 results in a shift of the charge transfer band to 383 nm (Figure 1) (17). Beside this difference, the d-d regions are almost identical in shape and intensity at low and high stoichiometry of Co(II) relative to the enzyme. A similar H-site occupancy at low and high stoichiometry reflects a strong preference of Co(II) for the H-site in Co1-BcII (17). Binding of D-Captopril to Co-BcII leads to changes both in the charge transfer region and in the d-d region indicating the binding of an additional ligand and likely changes in the coordination geometry of both sites. The difference spectra between inhibitor-bound and free enzyme at low and high stoichiometry are very similar in the charge transfer region and virtually identical in the d-d region indicating that the modes of binding for D-Captopril to Co(II) also are almost identical at both metal stoichiometries (Figure 1). It has to be emphasized, however, that even under the conditions used for the Co2-BcII experiments (Fig. 1A) the enzyme was not completely available as the Co2-species. Even at the very high concentration of Co(II) used a fraction of the enzyme still shows the charge transfer band for the mono-nuclear enzyme at 348 nm and thus 10-20% of the enzyme still had Co(II) bound only in the DCH-site. Thus, a direct quantitative comparison of absorption coefficients of Co1- and Co2- enzyme is difficult and, hence, a quantitative estimate of relative occupancies of both binding sites for the mononuclear enzyme.

EXAFS spectroscopy

For EXAFS spectroscopy on BcII and CphA approximately 0.8 eq of Cd per enzyme were used to minimise contributions from the eventually formed binuclear species. A three-fold surplus of D-Captopril was added in order to maximise the abundance of the inhibited species. The EXAFS results are given in Table 2 and Table 3 for BcII and CphA, respectively. The corresponding spectra are shown in Fig. 2.

To illustrate the fitting procedure used, Table 2 shows two alternative models used for simulation of the spectra for uninhibited Cd1-BcII, namely a 1-cluster and a 2-cluster model. Since it is
well-known that a single cadmium ion is distributed between the two available binding sites (10, 18) a typical 1-cluster model necessarily results in an “averaged” ligand sphere. From the coordination numbers resulting for the 1-cluster model it becomes obvious that neither the 3-His- nor the DCH-site is fully occupied (N_S ≈ 0.4; N_N/imidazole ≈ 2.02). The fractional occupation of the DCH-site (~40%) leads to a theoretical value of ~ 2.2 for N_N/imidazole which is in contradiction to the simulated value of N_N/imidazole ≈ 2.02. The resulting lack of intensity in the theoretical spectrum is compensated by an added broad contribution of O-ligands (high N_O and very high Debye-Waller factor) in this simple model. If multiple scattering contributions of histidines are not taken into account, the result becomes even more corrupted by not using constrained imidazole ring units since contributions from N- or O-ligands are virtually identical (data not shown). Also second shell contributions were omitted which is clearly reflected in the lack in intensity at ~ 3.2 Å (Figure 2C) of the theoretical spectrum.

Since the amino acid ligand geometry is well known for BcII from x-ray crystallographic data (pdb code 1BVT) we could make use of these informations by constructing a 2-cluster model. The atomic distribution in the 2 cluster model was accordingly assigned (3His- and DCH-site respectively). Thus the problem of correlated coordination numbers and Debye-Waller factors could be solved by fixing the ligand coordination numbers according to the structural data derived from the pdb file 1BVT. Thus the only free remaining coordination number in the 2-cluster model is the one of the Cd ion itself which determines the fractional occupation of each cluster. Additionally second shell N/O ligands with a fixed coordination number were introduced to account for back scattering contributions in the ~ 3.2 Å range. For the 2-cluster model an occupation of 70 % results for the 3H-site. Interestingly the ligand specific distances found are in good agreement with the values obtained with the 1-cluster model.

All attempts to generate theoretical spectra with 1-cluster models for the D-Captopril-inhibited enzyme (with exactly the same method as used for the uninhibited enzyme) failed in the sense that useful data could not be obtained, due to mutual dependences of parameters. Although low R-factors could be obtained, Debye-Waller factors and coordination numbers resulted in un-realistic values (data not shown). In the theoretical 2-cluster model for the D-captopril-inhibited enzyme it proved to be possible to replace the second shell N/O contributions in the DCH-site by introducing a small
molecule model of the Asp side chain. Again all coordination numbers except the coordination number of Cd(II) in both available binding sites were fixed. The resulting distribution of Cd(II) for the inhibited species shows a 60 % occupation of the DCH-site.

Table 2

The ligand geometry of Cd1-BcII is roughly similar to the one published for Zn1-BcII (17) [X-ray structure 1BVT (26)]. However, instead of one bound OH− as found in the Zn-EXAFS, the Cd-EXAFS shows two O-ligands at 2.17 Å ± 0.01 Å. This distance appears too long to be qualified as two hydroxide ions bound even taking the higher ionic radius of Cd into account. Together with the three amino acid ligands these two water molecules lead to a penta-coordinated H-site whereas the tetra-coordinated DCH-site is conserved relative to the zinc enzyme.

The metal distribution found for Cd1-BcII (70% of Cd are located in the H-site; 30% of Cd in the DCH-site) compares very well to the preference for the H-site suggested for cobalt but differs from the distribution found for the mono-zinc species where both sites are equally populated (17). The EXAFS spectrum from CphA in the presence of 0.8 eq. of Cd relative to the enzyme, here denoted Cd1-CphA can be fitted with only the DCH-site occupied and the fit gives one additional O-ligand very similar to the one found in Cd1-BcII.

Binding of D-Captopril to Cd1-BcII shifts the metal occupancy between the two sites to 40% in the H-site and 60% in the DCH-site. The sulphur of D-Captopril binds to the H-site and replaces the two previously bound water molecules. Both binding spheres appear tetra-coordinated. The additional oxygen ligand found in the DCH-site might be the carboxylate from D-Captopril. Figure 3B presents a hypothetical model where D-Captopril binds either with its thiolate sulphur to the Cd ion in the H-site, or with its carboxylate oxygen to the Cd ion when bound in the DCH-site. This assumption is based on the significantly shorter Cd-O distance in the inhibited complex (2.12 Å compared to the Cd-O distance of 2.28 Å in the Cd1 uninhibited enzyme).

In case of Cd1-CphA, D-Captopril binds to cadmium in the DCH-site with its thiolate sulphur, replacing the previously bound water molecule. There is no indication for any distribution of the Cd-
ion between the two sites, supported by the high rigidity (second sphere atoms are detectable with reasonable accuracy). This result is consistent with XAS studies on Zn₁-CphA (27).

For both inhibited enzyme species the Debye-Waller factors of sulphurs in the DCH-site are higher than those found for the uninhibited species. A possible explanation is, that binding of an additional negatively charged ligand to the DCH-site cadmium ion (captopril sulphur for CphA; a very close oxygen for BcII) results in a partial displacement of the bound Cys sulphur due to electrostatic repulsion. Thus the increased Debye-Waller factors might either result from an overestimation of the coordination number of sulphur which has been introduced by the chosen model, or by a “true” flexibility concerning the positioning of ligands. Due to the strong correlation of occupation number and Debye-Waller factor, however, an independent fitting of these parameters delivered no reliable results (data not shown).

PAC spectroscopy

PAC experiments on Cd-BcII derivatives with either 0.2 eq. Cd (Cd₁-BcII) or 1.7-1.8 eq. Cd (Cd₂-BcII) in the presence of either 1 eq. L-captopril or 1 eq. D-captopril show that 2 NQIs with nearly equal abundance can be detected in all cases (Table 4, Figure 4). The two NQI’s for both the L and D form of captopril as well as the two NQI’s for Cd₁- and Cd₂-BcII are different. PAC data for CphA are only shown for [Cd(II)]/[enzyme] stoichiometries below 1 (Table 4). The spectrum of the uninhibited CphA is characterised by two NQI’s with an abundance of 74% for the dominating form. In order to assign the two different NQIs to the two different metal sites an experiment with Moxalactam-modified enzyme was performed. A pre-treatment with Moxalactam leads to a covalent modification of the cysteine in the DCH site (7) whereby only the H site remains available for metal binding. One NQI with (ω₀=130 Mrad/s and η=0.6) now dominates the spectrum. Its close resemblance with the less populated NQI in the free enzyme leads to the conclusion that the DCH site of the uninhibited Cd₁-enzyme is populated to about 80%.

For Cd₁-CphA in the presence of 1 eq. of D-captopril PAC spectroscopy shows a single sharp NQI which is different from both NQI’s present without the inhibitor. For L-captopril under the same
conditions a single new NQI is detected being different from both the NQI in the presence of D-captopril and the two NQI’s detected without inhibitor.

Discussion

For the treatment of infectious diseases caused by beta-lactam-antibiotic resistant bacterial strains the availability of potent metallo-beta-lactamase inhibitors appears essential. Since all members of that enzyme class show two zinc binding sites in close proximity, the development of inhibitors is still focused on the binuclear enzymes (5;6). Structural models of the active sites are shown in Fig. 3. They are based on the EXAFS results presented on the cadmium enzymes and compared to previously obtained results on the Zn$_1$-BcII from EXAFS data (17).

Both the cadmium and zinc enzymes of BcII exhibit negative cooperativity with respect to metal binding to the two conserved sites (17;18). This, however, does not mean that there is a high and a low affinity binding site. We have shown earlier, that a single metal ion when bound to the “binuclear” site is distributed between both binding sites (10;12) and that rapid exchange among binding sites occurs (18). A recent work (28) shows that only mono-nuclear metallo-beta-lactamases might be physiologically important. These findings pose the question whether strategies to find inhibitors for the binuclear enzymes are the only ones being adequate. A set of structures has been obtained for inhibitors with a mercapto group coordinating with the sulphur to both zinc ions (4;5). In order for these inhibitors to be pharmaceutically relevant one needs to ensure that the inhibition constants are low for all metallo-beta-lactamases from pathogenic bacteria even at low zinc abundance. Under such conditions the mononuclear enzymes are the dominating form.

Whereas D- and L-captopril show identical inhibition constants for Zn$_1$- and Zn$_2$-BcII, within experimental error, both inhibitors show a higher efficiency for the Cd$_1$- compared to the Cd$_2$-species (compare Table 1). Since the two macroscopic dissociation constants for cadmium binding to BcII in the presence of D-Captopril differ by more than four orders of magnitude we mainly focused on inhibition of the mono-nuclear enzymes. Both EXAFS- and PAC-spectroscopy with Cd$_1$-BcII resulted in a distribution of the single metal ion between both binding sites. This has also been observed for
Zn₁-BcII (17). Surprisingly binding of D-Captopril does not result in a forced location of the metal ion in one of the two binding sites but leads only to a shift in the relative occupation.

The analysis of the EXAFS data (Table 2) further shows that the sulphur of D-captopril coordinates to Cd(II) when located at the H-site of BcII. For the fraction where the single cadmium ion is located in the DCH-site the carboxylate group of D-Captopril might be a ligand (see results section). The spectroscopic results obtained for Co₂-BcII (Figure 1) give further evidence for the binding of the D-Captopril sulphur at the H-site. The spectra of Co₁- and Co₂-BcII-D-Captopril complexes both show identical features in the d-d region which are significantly different from those of the uninhibited enzyme. It is likely that a replacement of water by sulphur in the H-site results in the observed changes (compare (29)). The appearance of two additional bands at 310 nm and 375 nm can be attributed to sulphur-Co LMCT due to binding of the Captopril sulphur. We did not perform an inhibition study of the Co(II)-enzymes since they show a reduced stability compared to the zinc and cadmium enzyme which results in unreliable steady-state kinetic data.

The weak binding of the second metal ion to the enzyme in presence of Captopril leads to the conclusion that the binuclear species might be of minor relevance as a target for inhibition with captopril-like compounds.

The enzyme from *Aeromonas hydrophila* is a representative of subclass B2 with a strong preference for Imipenem as substrate. Furthermore, the activity of the binuclear enzyme is reduced relative to the mononuclear enzyme (13). The main difference in the active site relative to BcII is a substitution of a His residue with an Asn residue in the H-site. The PAC results for Cd₁-CphA clearly demonstrate a distribution of the single Cadmium ion bound between both binding sites which are different from the one found for Cd₁-BcII (18). However, the DCH site is strongly preferred. PAC spectroscopy detects a single sharp coordination geometry for cadmium in the D-captopril-inhibited enzyme. The binding site of the cadmium ion is clearly identified as the DCH site by EXAFS spectroscopy. In case of the L-Captopril inhibited Cd-CphA a new unique coordination geometry is found by PAC spectroscopy. A strongly increased Gaussian distribution of the observed frequencies (Table 4) demonstrates a more flexible environment than the one found for the D-Captopril-inhibited species. Because of the nearly identical position of the first peak in the Fourier transform (ω₀) it is
likely that the same set of ligands composes the first coordination sphere of Cd(II) in L- and D-Captopril inhibited species. The difference in the symmetry parameters (η) (Table 4) is indicative for a modified geometrical arrangement of the ligands. For both enzymes studied, PAC derives a more rigid coordination geometry for the mononuclear cadmium enzyme with D-Captopril relative to L-Captopril as the inhibitor, consistent with lower inhibition constants for D-Captopril compared to L-Captopril (Table 1). Cd1- and Zn1-CphA clearly discriminate both diastereomers with a strong preference for D-Captopril whereas the inhibition constants for the binuclear form of all metal-substituted BeII species are very similar for D- and L-Captopril (Table 1).

Conclusions

For the purposes of the present investigation, the cobalt forms of the beta-lactamases have been utilised in the spectroscopic studies and the cadmium (isotope) forms for PAC, replacing zinc as the active site metal cofactor. The latter is, by convention, assumed to be the metal cofactor form(s) of functional importance. Nonetheless, this is offset by the fact that, in contrast to the solid state environment of X-Ray crystallography, the use of Co and Cd as probes in the present inhibition studies permitted the investigation to be conducted in solution, a situation that closely parallels that of the real environment of a working enzyme. It is not unreasonable to consider that the conclusions that we have drawn for the Co and Cd enzyme forms may also be applicable for situations involving the zinc enzyme. The structural investigation of the two enzymes from different subclasses leads to different inhibitor binding modes. This observation indicates that it might be very difficult to develop single inhibitors which are able to fight all metallo-beta-lactamase subclasses. In particular, the relative importance of the mononuclear versus the binuclear species for a specific inhibitor needs to be addressed. The enzyme-specific binding and intramolecular mobility of the metal ion might also be essential elements for the understanding of both the broad substrate profiles and the catalytic mechanisms and thereby for the design of effective inhibitors.

Acknowledgements

We thank Dr. B. Wannemacher for help with the atomic absorption measurements and Marianne Lund Jensen for excellent laboratory work during the PAC experiments.
Reference List


Footnotes

†The abbreviations used are: PAC, Perturbed Angular Correlation of γ-rays; EXAFS, Extended X-ray Absorption Fine Structure; BcII, metallo-β-lactamase from Bacillus cereus; H-site, zinc binding site composed of three histidine residues in subclass B1; DCH-site, zinc binding site composed of asparagine, cysteine and histidine; NQI, Nuclear Quadrupole Interaction; NMR, Nuclear Magnetic Resonance; LMCT, ligand-to-metal charge transfer, MF, Mag-fura-2
Legends to the Schemes

**Scheme 1:** Synthetic pathway used for the synthesis of D-Captopril.

(a) SOCl₂, pyridine, toluene; (b) D-proline, Et₃N, dioxane, H₂O ; (c) (i) NaOH 1N, MeOH, (ii) HCl

Legends to the figures

**Figure 1:** Binding of D-Captopril to Co(II)-substituted BcII monitored by UV-vis spectroscopy.

A.: Co₂-BcII. The Co-species (118 µM apo-BcII + 500 µM Co(II)) is represented as bold full line, the D-Captopril-inhibited species (addition of 800 µM D-Captopril) as dotted line and the difference spectrum as thin full line.

B.: Co₁-BcII. The mono-Co-species (130 µM apo-BcII + 120 µM Co(II)) is represented as bold full line, the D-Captopril inhibited species (addition of 800 µM D-Captopril) as dotted line and the difference spectrum as thin full line. Spectra were recorded in 15 mM HEPES, pH 7.

**Figure 2:** EXAFS results for Cd₁-BcII and Cd₁-AER with and without D-Captopril. The k²-weighted EXAFS spectra (open circles) with the theoretical fits (full line) are shown to the left. The corresponding Fourier transforms in R-space (experimental data and fit represented as open circles and full line, respectively) are shown to the right. The fitting parameters and details of the fitting procedure are listed in Tables 2 and 3. Up to 30 scans/sample (taken at a temperature of 25 K) were averaged. In the Fourier transform of spectrum C an alternative fit with a 1-cluster model (compare Table 2) is shown with a crosshair line, resulting in a clearly missing amplitude in the 3.2 Å range.

**Figure 3:** Structural models of the BcII and CphA (AER ae036) active sites based on the EXAFS results.

A: Active site models for both Cd₁-BcII and –CphA.

B: Active site models for both the D-Captopril-inhibited species of Cd₁-BcII and Cd₁-CphA.
C: Active site model of Zn$_1$-BcII (17) and the two diastereomers D- and L-Captopril to the right.

**Figure 4:** *PAC results both for BcII and CphA.*

The experimental data are shown as dotted line with the corresponding fit as full lines.

A. BcII with 0.2 eq Cd and 1 eq L-Captopril
B. BcII with 0.2 eq Cd and 1 eq D-Captopril
C. BcII with 1.8 eq Cd and 1 eq L-Captopril
D. BcII with 1.7 eq Cd and 1 eq D-Captopril
E. CphA with 0.2 eq Cd
F. CphA with 0.2 eq Cd and 1 eq D-Captopril
G. CphA with 0.2 eq Cd and 1 eq L-Captopril
Tables

**Table 1**: Enzymatic activities with imipenem and inhibition constants of D- and L-Captopril for Zn- and Cd-substituted BcII and CphA.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Metals</th>
<th>$K_M$ [µM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat}/K_M$ [s$^{-1}$µM$^{-1}$]</th>
<th>$K_I$ D-Captopril [µM]</th>
<th>$K_I$ L-Captopril [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCII</td>
<td>Zn$_1$</td>
<td>73 ± 10</td>
<td>127 ± 7</td>
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<td>44 ± 5</td>
<td>61 ± 5</td>
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<tr>
<td></td>
<td>Zn$_2$</td>
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<td>276 ± 20</td>
<td>0.84</td>
<td>45 ± 5</td>
<td>65 ± 5</td>
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<tr>
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<td>Cd$_1$</td>
<td>820 ± 39</td>
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<tr>
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<td>0.00068</td>
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<td>25 ± 3</td>
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<tr>
<td>CphA</td>
<td>Zn$_1$</td>
<td>100$^\dagger$</td>
<td>625$^\dagger$</td>
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<td>950 ± 80</td>
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<tr>
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<td>Cd$_1$</td>
<td>35.5$^\dagger$</td>
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<td>19 ± 2</td>
</tr>
</tbody>
</table>

$^\dagger$ Data published in (30).
Table 2: Results of the theoretical EXAFS data analysis for BcII.

Histidine ligands were treated as imidazole units with N as the pivotal atom. Debye Waller factors were constrained: \(a^*\) was constrained to be equal to \(a\) (same procedure for \(b^*/b\), \(c^*/c\)).

<table>
<thead>
<tr>
<th>Site/Cd</th>
<th>ligand</th>
<th>atom</th>
<th>(N_i)</th>
<th>(R_i)</th>
<th>Debye Waller factor</th>
<th>(E_f)</th>
<th>(R_{EXAFS})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd(_{1})-BcII, 1 cluster</td>
<td>-/1</td>
<td>His</td>
<td>N</td>
<td>2.02±0.42</td>
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<td>-4.8 ± 0.31</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>0.023±0.011</td>
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</tr>
<tr>
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<td></td>
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<td>C</td>
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<td></td>
<td>0.023±0.011</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>4.47</td>
<td></td>
<td>0.012±0.006</td>
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</tr>
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<td>H(_2)O/OH(^-)</td>
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</tr>
<tr>
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<td>Cys</td>
<td>S</td>
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<td>0.001±0.003</td>
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### Table 3: Results of the theoretical EXAFS data analysis for CphA

Histidine ligands were treated as imidazole units with N as the pivotal atom. Debye Waller factors were constrained: $a^*$ was constrained to be equal to $a$ (same procedure for $b^*/b$, $c^*/c$).

<table>
<thead>
<tr>
<th>ligand</th>
<th>atom</th>
<th>$N_i$</th>
<th>$R_i$</th>
<th>Debye Waller factor</th>
<th>$E_f$</th>
<th>$R_{EXAFS}$</th>
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<tr>
<td><strong>Cd$_1$-CphA</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>S</td>
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<td>2.515 ± 0.015</td>
<td>0.005 ± 0.002$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>N</td>
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<td>2.363 ± 0.037</td>
<td>0.005 ± 0.002$^a^*$</td>
<td>-1.67 ± 1.05</td>
<td>40.83</td>
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<td>C</td>
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<td>3.195</td>
<td>0.008 ± 0.021$^b*$</td>
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<td>4.410</td>
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<td>4.218</td>
<td>0.021 ± 0.017$^b*$</td>
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<td>2.253 ± 0.015</td>
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<td>0.02 ± 0.30</td>
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<td>second sphere</td>
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<td>3.574 ± 0.025</td>
<td>0.010 ± 0.006$^a$</td>
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<td>O/N/C</td>
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<td>3.016 ± 0.053</td>
<td>0.027 ± 0.019</td>
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<td></td>
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</tbody>
</table>

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Table 4: PAC results for D- and L-Captopril inhibited BcII and CphA at different Cd-stoichiometries and the data for the uninhibited and moxalactam-inhibited species of CphA.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>Cd-eq</th>
<th>NQI</th>
<th>amp</th>
<th>η</th>
<th>ω₀</th>
<th>δ</th>
<th>1/τ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BcII</td>
<td>L-Captopril</td>
<td>0.2</td>
<td>1</td>
<td>61 ± 4</td>
<td>0.38 ±0.02</td>
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<td>0.11 ±0.01</td>
<td>111 ± 7</td>
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<tr>
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<td></td>
<td>2</td>
<td>39 ± 4</td>
<td>0.58 ±0.03</td>
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<tr>
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<td></td>
<td>1.8</td>
<td>60 ± 3</td>
<td>0.48 ±0.01</td>
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<tr>
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<td>49 ± 3</td>
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<td>51 ± 3</td>
<td>0.65 ±0.01</td>
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<td>CphA</td>
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<td>9 ± 3</td>
<td>0.31 ±0.25</td>
<td>72 ± 10</td>
<td>0.10 ±0.01</td>
<td>83 ± 7</td>
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<td></td>
<td>2</td>
<td>91 ± 3</td>
<td>0.40 ±0.01</td>
<td>131.6 ±0.8</td>
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<td>-</td>
<td></td>
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<tr>
<td>D-Captopril</td>
<td>0.2</td>
<td>1</td>
<td>100</td>
<td>0.26 ±0.01</td>
<td>178.9 ±0.6</td>
<td>0.03 ±0.01</td>
<td>91 ± 8</td>
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<tr>
<td>L-Captopril</td>
<td>0.2</td>
<td>1</td>
<td>100</td>
<td>0.58 ±0.01</td>
<td>146.2 ±1.3</td>
<td>0.12 ±0.01</td>
<td>250 ±63</td>
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</tbody>
</table>
Schemes:

**Scheme 1**: *Synthetic pathway used for the synthesis of D-Captopril.*

(a) SOCl₂, pyridine, toluene; (b) D-proline, Et₃N, dioxane, H₂O; (c) (i) NaOH 1N, MeOH, (ii) HCl
Figures

Figure 1

![Absorbance vs Wavelength Graphs](image-url)
Figure 2:

The figure shows graphs of the pair correlation function, $g(r)$, and its second derivative with respect to wavevector, $k^2 g(k)$, for various complexes.

- **A**: $Cd_1$-CphA
- **B**: $Cd_1$-CphA-D-captopril
- **C**: $Cd_1$-BclI
- **D**: $Cd_1$-BclI-D-captopril

The graphs are plotted against the wavevector $k$ (in Å$^{-1}$) on the left and the distance $r$ (in Å) on the right. The y-axis represents the $k^2 g(k)$ values in arbitrary units.
### Figure 3

<table>
<thead>
<tr>
<th>Bell</th>
<th>CphA</th>
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<tr>
<td><strong>A</strong></td>
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<tr>
<td><strong>B</strong></td>
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<tr>
<td><img src="image5" alt="Chemical Structure" /></td>
<td><img src="image6" alt="Chemical Structure" /></td>
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</tbody>
</table>

D-Captopril  
L-Captopril
Figure 4:

![Graph 1](attachment://Graph1.png)

- **A** Cd1-BclI-L-captopril
- **B** Cd1-BclI-D-captopril
- **C** Cd2-BclI-L-captopril
- **D** Cd2-BclI-D-captopril
- **E** Cd1-CphA
- **F** Cd1-CphA-L-captopril
- **G** Cd1-CphA-D-captopril

2D Graph 6

arbitrary units

Grad/s

0.2 0.4 0.6

0.0 0.5 1.0 1.5 2.0

0.2 0.4 0.6

0 1 2 3

0 1 2 3
Additions and Corrections


Coordination geometries of metal ions in D- or L-captopril-inhibited metallo-β-lactamases.

Uwe Heinz, Rogert Bauer, Sandra Wommer, Wolfram Meyer-Klaucke, Cyril Papamicaël, John Bateson, and Hans-Werner Adolph

Dr. Papamicaël’s name was listed incorrectly. The correct spelling is shown above.
Coordination geometries of metal ions in D- or L-captopril-inhibited metallo-beta-lactamases
Uwe Heinz, Rogert Bauer, Sandra Wommer, Wolfram Meyer-Klaucke, Cyril Papamichaels, John Bateson and Hans-Werner Adolph

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