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Substrate-activated Zinc Binding of Metallo-β-lactamases

PHYSIOLOGICAL IMPORTANCE OF THE MONONUCLEAR ENZYMES*

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Sandra Wommer‡, Sandrine Rival‡, Uwe Heinz‡, Moreno Galleni§, Jean-Marie Frère§, Nicola Franceschini¶, Gianfranco Amicosante¶, Bodil Rasmussen‖, Rogert Bauer‡‡, and Hans-Werner Adolph¶¶‖‖**

*From the ‡Department of Biochemistry, University of Saarland, D-66041 Saarbrücken, Germany, the §Centre d'Ingenierie des Proteines, Université de Liège, Sart Tilman, 4000 Liège, Belgium, the ¶Dipartimento di Scienze e Technologie Biomediche e di Biometria, Universita dell' Aquila, 67100 L'Aquila, Italy, the Departments of ‖Chemistry and **Mathematics and Physics, Royal Veterinary and Agricultural University, DK-1871 Frederiksberg C, Denmark, and the ¶¶Center for Bioinformatics, University of Saarland, D-66041 Saarbrücken, Germany and the Max-Planck Institut für Informatik, D-66123 Saarbrücken, Germany*

We have investigated the influence of substrate binding on the zinc ion affinity of representatives from the three metallo-β-lactamase subclasses, B1 (BcII from *Bacillus cereus* and BlaB from *Chryseobacterium meningosepticum*), B2 (CphA from *Aeromonas hydrophila*), and B3 (L1 from *Stenotrophomonas maltophilia*). By competition experiments with metal-free apoenzymes and chromophoric zinc chelators or EDTA, we determined the dissociation constants in the absence and presence of substrates. For the formation of the monozinc enzymes we determined constants of 1.8, 5.1, 0.007, and 2.6 nM in the absence and 13.6, 1.8, 1.2, and 5.7 pM in the presence of substrates for BcII, BlaB, CphA, and L1, respectively. A second zinc ion binds in the absence (presence) of substrates with considerably higher dissociation constants, namely 1.8 (0.8), 0.007 (0.025), 50 (1.9), and 0.006 (0.12) μM for BcII, BlaB, CphA, and L1, respectively. We have concluded that the apo form might be the prevailing state of most of the metallo-β-lactamases under physiological conditions in the absence of substrates. Substrate availability induces a spontaneous self-activation due to a drastic decrease of the dissociation constants, resulting in the formation of active mononuclear enzymes already at picomolar free zinc ion concentrations. In the presence of substrates, the binuclear state of the enzymes only exists at unphysiologic high zinc concentrations and might be of no biological relevance. From the competition experiments with EDTA it is further concluded that the reactivation rate does not depend on the pool of free zinc ions but proceeds via the EDTA-Zn(II)-enzyme ternary complexes.

Metallo-β-lactamases (class B β-lactamases) are produced by bacteria as extracellular or periplasmatic enzymes. All known representatives possess two conserved metal binding sites and require zinc ions as enzymatic cofactors. By catalyzing the hydrolysis of β-lactams they render the corresponding strains resistant to almost all β-lactam antibiotics. Their increasing emergence in pathogenic bacterial strains due to a rapid dissemination by horizontal gene transfer has induced a growing interest in this enzyme family because of the lack of efficient therapies to treat infected patients.

The metallo-β-lactamases constitute a group of heterogeneous proteins that is divided into subclasses B1, B2, and B3 (1). Subclass B1 exhibits a broad substrate profile (2), and its zinc binding sites are composed of His-116, His-118, and His-196 (site 1) and Asp-120, Cys-221, and His-263 (site 2) (numbering according to Ref. 3). In subclass B2 the zinc ligands in site 2 are conserved, whereas His-116 in site 1 is replaced by Asn. Representatives of subclass B2 efficiently hydrolyze only carbapenems (2) and are active as monozinc enzymes, whereas the binding of a second zinc ion causes non-competitive inhibition (4). In subclass B3, Cys-221 is substituted by a Ser and is replaced by His-121 as a zinc ligand in site 2. In the Gob-1 enzyme (*Chryseobacterium meningosepticum* PINT) an additional His-116 → Gln mutation has been reported. Subclass B3 enzymes exhibit a broad spectrum activity profile with a putative preference for cephalosporins (5). The question as to whether zinc-β-lactamases are active as the mono- or the dizinc enzyme has been controversially discussed, and two alternative mechanisms for both enzyme states have been proposed (6, 7).

The known dissociation constants of class B β-lactamases for the formation of monozinc enzymes range from 6 pM for CphA[1] (8) to 0.6 nM for BcII, strain 569/H/9 (9). Others (10) report dissociation constants in the micromolar range for BcII. A second zinc ion is bound with dissociation constants in the micromolar range (8, 9). In particular, the low affinity for a second zinc ion leads to the question of whether or not physiological conditions offer sufficiently high metal ion concentrations to maintain the active metal-bound state of the enzymes. Recently, it was shown that the available concentration of free zinc ions in eukaryotic cells is controlled by the metallothionein/thionein pair (11). The presence of free thionein in different cell types (11) combined with the picomolar dissociation constant of metallothionein (12) implicate free zinc ion concentrations in the picomolar range. A regulation system involving metalloregulatory proteins was recently evidenced in bacteria

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‖‖ To whom correspondence should be addressed. Tel.: 49-681-302-2492; Fax: 49-681-302-2097; E-mail: hwadolph@rz.uni-sb.de.

[1] The abbreviations used are: CphA, metallo-β-lactamase from *Aeromonas hydrophila*; BcII, metallo-β-lactamase from *Bacillus cereus*; BlaB, metallo-β-lactamase from *Chryseobacterium meningosepticum*; L1, metallo-β-lactamase from *Stenotrophomonas maltophilia*; MF, Mag-fura-2.

(13), resulting in estimated femtomolar concentrations of free cytosolic zinc. For the operational area of metallo-β-lactamases (periplasm or the extracellular environment of bacteria) similarly low concentrations of free zinc might be assumed because of the strong competition of various bacterial proteins for the metal ions, especially under conditions of bacterial growth. Physiological conditions would therefore not fulfill the requirements for a metal-bound enzyme state.

A first indication of a substrate-modified affinity for zinc ions resulted from a study (14) of BcII from strain 5/B/6, whose apoenzyme shows a spontaneous reactivation in the presence of the substrate nitrocefin in a medium without added zinc. In the present study we investigated the influence of substrates on the zinc ion affinity of representatives of subclasses B1 (BcII from *Bacillus cereus* 569/H/9 and BlaB from *C. meningosepticum* NCTC10585), B2 (CphA from *Aeromonas hydrophila*), and B3 (the only known tetrameric enzyme, L1, from *Stenotrophomonas maltophilia*).

MATERIALS AND METHODS

Enzymes and Substrates—The metallo-β-lactamases CphA from *A. hydrophila* AE036, BcII from *B. cereus* 569/H/9, L1 from *S. maltophilia*, and BlaB from *C. meningosepticum* NCTC10585 were produced and purified as described in Refs. 15, 14, 16, and 17, respectively. The protein concentrations were determined with the following extinction coefficients: $\epsilon_{280(\mathrm{BcII})}$ = 30,500 $\mathrm{M^{-1}\ cm^{-1}}$, $\epsilon_{280(\mathrm{CphA})}$ = 38,000 $\mathrm{M^{-1}\ cm^{-1}}$, $\epsilon_{280(\mathrm{L1})}$ = 55,000 $\mathrm{M^{-1}\ cm^{-1}}$ (per monomer), and $\epsilon_{280(\mathrm{BlaB})}$ = 45,670 $\mathrm{M^{-1}\ cm^{-1}}$.

The carbapenem antibiotic imipenem (Merck Sharp & Dohme) was used as a substrate for all enzymes studied except for BlaB. Hydrolysis was followed at 300 nm using $\Delta\epsilon_{300}$ = −9,000 $\mathrm{M^{-1}\ cm^{-1}}$. BlaB was studied with nitrocefin (Unipath Oxoid, Basingstoke, UK) as the substrate using $\Delta\epsilon_{482}$ = 15,000 $\mathrm{M^{-1}\ cm^{-1}}$ to follow the hydrolysis.

To minimize zinc ion contamination, buffer solutions were prepared in bidistilled water and extensively stirred with Chelex 100 (Sigma). Zinc concentrations in apoenzyme samples were measured with a PerkinElmer 2100 atomic absorption spectrometer in the flame mode at protein concentrations of 50–100 μM as described (8).

The apoenzymes of BcII and L1 were prepared by three dialysis steps of the corresponding enzymes (2 and 10 mg/ml, respectively) against a 250-fold excess of 15 mM HEPES, pH 7.0 and 6.5, respectively, containing 20 mM EDTA (24 h each with stirring at 4 °C). EDTA was removed by three dialysis steps against the same buffer containing 1 M sodium chloride followed by two steps without salt or with 0.15 M NaCl for BcII and L1, respectively. Zinc-free CphA was obtained similarly by dialysis against three changes of 10 mM EDTA in 15 mM HEPES, pH 6.5. EDTA was removed by six dialysis steps against 15 mM HEPES, pH 6.5, containing 0.15 M NaCl for the first two dialysis steps. Apo-BlaB was produced by three dialysis steps of the enzyme (1.3 mg/ml) against a 150-fold volume excess of 30 mM sodium cacodylate, pH 6.5, containing 20 mM EDTA, 0.1 M NaCl (12 h each with stirring at 4 °C). EDTA was removed by dialysis against three changes of a 150-fold volume excess of 30 mM sodium cacodylate, 1.0 M NaCl, pH 6.5, followed by two changes of the same buffer containing 0.1 M NaCl (or without NaCl). In all apoenzyme preparations the remaining zinc content did not exceed 1–7.5%.

Determination of Metal Ion Affinities in the Absence and Presence of Substrates—The dissociation constants for a first (K_{mono}) and a second zinc ion (K_{bi}) bound in the absence of substrates were obtained from competition experiments with the chromophoric zinc chelator Mag-fura-2 (MF) (Molecular Probes, Eugene, OR). The method is based on the different spectroscopic characteristics of MF and the Zn-Mag-fura-2 complex (Zn-MF). The dissociation constant of Zn-MF and the absorption coefficient of MF at 363 nm were determined from titrations with zinc ions under the conditions used in the competition experiments and resulted in $K_{\mathrm{Zn\text{-}MF}}$ = 28 nM and $\epsilon_{(\mathrm{MF,363\ nm})}$ = 28,500 $\mathrm{M^{-1}\ cm^{-1}}$.

The competition experiments with apoenzymes were performed at protein/MF ratios between 1:1 and 1:3 following the total absorbance at 363 nm, which is composed of contributions from MF and Zn-MF according to Equation 1.

$$A_{total} = \varepsilon_{\mathrm{MF}}[\mathrm{MF}] + \varepsilon_{\mathrm{Zn\text{-}MF}}[\mathrm{Zn\text{-}MF}] \qquad \text{(Eq. 1)}$$

Solutions containing apoenzyme ($[E]_{\mathrm{start}}$) and a known concentration of MF ($[\mathrm{MF}]_{\mathrm{start}}$) were titrated with zinc and resulted in data that give the total absorbance (A_{total}) measured as a function of the added volume of a metal ion stock solution ($[\mathrm{Zn}]_{\mathrm{stock}}$) to a defined starting volume. For numerical data analysis we used the program Chemsim, which calculates simulated absorbance values for each titration step according to Equation 1. The bases for these calculations are the laws of mass action (Equations 2–4), the ionic product of water when needed (see the competition experiments with EDTA), and the equations for mass conservation.

$$\mathrm{MF} + \mathrm{Zn^{2+}} \xleftrightarrow{K_{(\mathrm{Zn\text{-}MF})}} \mathrm{Zn\text{-}MF}, \quad K_{Zn\text{-}MF} = \frac{[\mathrm{Zn^{2+}}][\mathrm{MF}]}{[\mathrm{Zn\text{-}MF}]} = 28\mathrm{nM} \qquad \text{(Eq. 2)}$$

$$\mathrm{E} + \mathrm{Zn^{2+}} \leftrightarrow \mathrm{ZnE},\ K_{mono} = \frac{[\mathrm{Zn^{2+}}][\mathrm{E}]}{[\mathrm{ZnE}]} = ?\mathrm{M} \qquad \text{(Eq. 3)}$$

$$\mathrm{ZnE} + \mathrm{Zn^{2+}} \leftrightarrow \mathrm{Zn_2}E,\ K_{bi} = \frac{[\mathrm{Zn^{2+}}][\mathrm{ZnE}]}{[\mathrm{Zn_2E}]} = ?\mathrm{M} \qquad \text{(Eq. 4)}$$

For data evaluation of competition experiments ϵ_{MF}, $[\mathrm{MF}]_{\mathrm{start}}$, $K_{\mathrm{Zn\text{-}MF}}$, starting volume, and $[\mathrm{Zn}]_{\mathrm{stock}}$ were constrained. In a least squares procedure, K_{mono}, K_{bi}, $[E]_{\mathrm{start}}$, and $\epsilon_{\mathrm{Zn\text{-}MF}}$ were simultaneously optimized. A more detailed description of the method can be found in Ref. 9.

For the determination of zinc dissociation constants of metallo-β-lactamases in the presence of substrates, we have developed a method based on steady-state rate determinations. The method makes use of the competition of the enzymes (E) with EDTA ($\mathrm{Y^{4-}}$) for the available zinc ions.

Under conditions where $[\mathrm{EDTA}]_{\mathrm{total}} \gg [E]_{\mathrm{total}}$, EDTA has the role of a zinc ion buffer by solely defining the free zinc ion concentration ($[\mathrm{Zn^{2+}}]_{\mathrm{free}}$) as a function of $[\mathrm{Zn}]_{\mathrm{total}}/[\mathrm{EDTA}]_{\mathrm{total}}$. The zinc binding characteristics of EDTA are strongly pH-dependent, because only the fully deprotonated compound ($\mathrm{Y^{4-}}$) has a strong affinity for zinc, whereas $\mathrm{HY^{3-}}$ and $\mathrm{H_2Y^{2-}}$ are the dominating protonation states at physiological pH. The dissociation constant of the $\mathrm{ZnHY^-}$ complex is already millimolar (Equation 12), and the binding of $\mathrm{Zn^{2+}}$ to $\mathrm{H_2Y^{2-}}$ is too weak to be considered. Additionally, the formation of $\mathrm{ZnOH^+}$ and the solubility product of $\mathrm{Zn(OH)_2}$ ($K_{\mathrm{sol,Zn(OH)_2}}$) significantly contribute to the distribution of zinc among different compounds. For a given $[\mathrm{Zn}]_{\mathrm{total}}/[\mathrm{EDTA}]_{\mathrm{total}}$ ratio, the following equilibria determine $[\mathrm{Zn^{2+}}]_{\mathrm{free}}$ (Equations 5–12).

$$\mathrm{Y^{4-}} + \mathrm{H^+} \leftrightarrow \mathrm{HY^{3-}},\ K_{a,HY^{3-}} = \frac{[\mathrm{H^+}][\mathrm{Y^{4-}}]}{[\mathrm{HY^{3-}}]} = 5.888 \times 10^{-11}\mathrm{M}$$

(Ref. 18) (Eq. 5)

$$\mathrm{HY^{3-}} + \mathrm{H^+} \leftrightarrow \mathrm{H_2Y^{2-}},\ K_{a,H_2Y^{2-}} = \frac{[\mathrm{H^+}][\mathrm{HY^{3-}}]}{[\mathrm{H_2Y^{2-}}]} = 6.309 \times 10^{-7}\ \mathrm{M}$$

(Ref. 18) (Eq. 6)

$$\mathrm{H_2Y^{2-}} + \mathrm{H^+} \leftrightarrow \mathrm{H_3Y^-},\ K_{a,H_3Y^-} = \frac{[\mathrm{H^+}][\mathrm{H_2Y^{2-}}]}{[\mathrm{H_3Y^-}]} = 2.138 \times 10^{-3}\ \mathrm{M}$$

(Ref. 18) (Eq. 7)

$$\mathrm{H_3Y^-} + \mathrm{H^+} \leftrightarrow \mathrm{H_4Y},\ K_{a,H_4Y} = \frac{[\mathrm{H^+}][\mathrm{H_3Y^-}]}{[\mathrm{H_4Y}]} = 1.023 \times 10^{-2}\mathrm{M}$$

(Ref. 18) (Eq. 8)

$$\mathrm{Zn^{2+}} + \mathrm{OH^-} \leftrightarrow \mathrm{ZnOH^+},\ K_{d,ZnOH^-} = \frac{[\mathrm{Zn^{2+}}][\mathrm{OH^-}]}{[\mathrm{ZnOH^+}]} = 1.122 \times 10^{-5}\ \mathrm{M}$$

(Ref. 19) (Eq. 9)

$$\mathrm{ZnOH^+} + \mathrm{OH^-} \leftrightarrow \mathrm{Zn(OH)_2},\ K_{sol,Zn(OH)_2} = \frac{[\mathrm{ZnOH^+}][\mathrm{OH^-}]}{[\mathrm{Zn(OH)_2}]} = 5.371 \times 10^{-11}\ \mathrm{M}$$

(Ref. 19) (Eq. 10)

$$\mathrm{Y^{4-}} + \mathrm{Zn^{2+}} \leftrightarrow \mathrm{ZnY^{2-}},\ K_{d,ZnY^{2-}} = \frac{[\mathrm{Zn^{2+}}][\mathrm{Y^{4-}}]}{[\mathrm{ZnY^{2-}}]} = 5.75 \times 10^{-17}\ \mathrm{M}$$

(Ref. 20) (Eq. 11)

$$\mathrm{HY^{3-}} + \mathrm{Zn^{2+}} \leftrightarrow \mathrm{ZnHY^-},\ K_{d,ZnHY^-} = \frac{[\mathrm{Zn^{2+}}][\mathrm{HY^{3-}}]}{[\mathrm{ZnHY^-}]} = 1.00 \times 10^{-3}\ \mathrm{M}$$

(Ref. 20) (Eq. 12)

TABLE I

Dissociation constants for the binding of zinc ions in the absence and presence of substrates and kinetic parameters for the Zn_1- and Zn_2-enzymes

Dissociation constants for the formation of the mononuclear (K_{mono}) and binuclear zinc enzymes (K_{bi}) in the absence and formation of active zinc enzymes in the presence of substrate ($K_{mono,sub}$ and $K_{bi,sub}$) are compared. Kinetic parameters for Zn_1-BcII were determined for an apoenzyme reconstituted with 0.1 equivalent of zinc, whereas Zn_2-BcII was studied in the presence of 10 μM zinc. Zn_1-BlaB was obtained from the apoenzyme by the addition of 0.6 equivalents of zinc, whereas Zn_2-BlaB was studied in the presence of 50 μM free zinc. Zn_1-L1 was reconstituted with 0.1 equivalents of zinc, and Zn_2-L1 was studied in the presence of 1 μM zinc. Calculations of k_{cat} are always based on the zinc-enzyme concentration. All values are the averages of three determinations; S.D., standard deviations are given in parentheses.

Enzyme	State	k_{cat}	K_m	k_{cat}/K_m	K_{mono}	$K_{mono,sub}$	K_{bi}	$K_{bi,sub}$
		s^{-1}	μM	$\mu M^{-1}\ s^{-1}$	pM	pM	μM	μM
BcII	Zn_1	127 (±7)	73 (±10)	1.74	1800 (±300)	13.6 (±5)	1.8 (±0.2)	0.8 (±0.2)
	Zn_2	276 (±20)	330 (±30)	0.84				
BlaB	Zn_1	22.2 (±0.5)	6.9 (±1.1)	3.2	5100 (±1500)	1.8 (±0.2)	0.007 (±0.002)	0.025 (±0.004)
	Zn_2	58 (±2)	7.2 (±1.0)	8.1				
CphA	Zn_1	1300 (625)[a]	180 (100)[a]	7.2 (6.3)	7[a]	1.2 (±0.2)	50[a]	1.9 (±0.3)
	Zn_2	240[a]	170[a]	1.4				
L1	Zn_1	11 (±3)	8 (±2)	1.4	2600 (±1000)	5.7 (±2.0)	0.006 (±0.002)	0.12 (±0.03)
	Zn_2	328 (±30)	73 (±6)	4.5				

[a] Data from Ref. 8. The experiments were performed in 30 mM sodium cacodylate, pH 6.5, 30 °C; values in parentheses were determined in 50 mM HEPES pH 7.5. Parameters for Zn_2-CphA were determined in the presence of 200 μM Zn(II).

If competition experiments with EDTA are used to determine K_{mono} and K_{bi}, Equations 3 and 4 have to be included additionally. For an experimental determination, a signal is required that systematically changes with a changing ratio of $[Zn]_{total}/[EDTA]_{total}$. We chose to monitor the changes of enzymatic activities.

Reactions were started by the addition of apoenzyme stock solutions to cuvettes containing the given concentrations of substrate, EDTA, and zinc. The apoenzymes were preincubated with EDTA to guarantee that no initial activity due to residual bound zinc was left. Activities were determined after steady-state conditions had been established. The reported values are the means of at least three independent determinations. All experiments were performed at 25 °C in 15 mM HEPES, pH 7.0, except those with BlaB, which were performed in 30 mM sodium cacodylate, pH 6.5.

The activities obtained at defined ratios of $[Zn]_{total}/[EDTA]_{total}$ (ν_{obs}) result from Equation 13.

$$\nu_{obs} = k_{app,ZnE}[ZnE] + k_{app,Zn2E}[Zn_2E] \quad \text{(Eq. 13)}$$

For all the enzymes investigated, the apparent specific activities of the Zn_1 and Zn_2 enzyme species ($k_{app,ZnE}$ and k_{app,Zn_2E}, respectively) appeared to be different. Conditions were chosen so that the experimentally varied range of $[Zn^{2+}]_{free}$ covered K_{mono} and K_{bi}. Thus, a series of activity measurements at different ratios of $[Zn]_{total}/[EDTA]_{total}$ could be treated like a titration and subjected to numerical data analysis. Data files were generated giving the activity measured (ν_{obs}) as a function of the added volume of a metal ion stock solution ($[Zn]_{stock}$) to a defined starting volume containing a known concentration of apoenzyme ($[E]_{start}$) and total EDTA ($[EDTA]_{start}$). In the numerical data analysis, Equations 3–12 defined the underlying equilibria, and Equation 13 was used to calculate the zinc concentration-dependent signal. Starting concentrations of apoenzyme, EDTA (or total zinc), and the proton concentration were constrained for the simultaneous optimization of K_{mono}, K_{bi}, $k_{app,ZnE}$, and k_{app,Zn_2E}. The same set of equations could be used for all experiments by changing the starting concentrations of total EDTA, zinc, and enzyme as well as pH to the appropriate values.

RESULTS AND DISCUSSION

Here we present a systematic investigation of the influence of substrates on the zinc dissociation constants of representatives of all three subclasses of metallo-β-lactamases. Dissociation constants for a first and a second zinc ion bound to BcII, BlaB, and L1 in the absence of substrate were determined in competition experiments with the chromophoric Zn(II) chelator Mag-fura-2. BlaB and L1 bind two zinc ions with very similar affinity, whereas BcII shows strong negative cooperativity for zinc binding (Table I). It was shown for cadmium-substituted BcII that the metal ion in the mononuclear enzyme moves between both binding sites in a microsecond time regime (21). A similar dynamic behavior might account for the negative cooperativity observed for zinc-bound BcII (9).

For all the apoenzymes studied we observed a spontaneous reactivation in the presence of substrates, although metal-depleted buffers were used. Whereas the reactivation of BcII is slow (Fig. 1*A*), CphA reaches full activity of imipenem conversion after 25 s (Fig. 1*B*). We have shown earlier (4) that metal-depleted buffers still contain up to 20 nM Zn(II). Such concentrations are sufficient to completely reconstitute CphA (8 nM in the experiment shown in Fig. 1) but not BcII (40 nM in the experiment). Therefore full reactivation of BcII could only be expected with added Zn(II). The addition of 100 μM EDTA to the reaction medium prevented the reactivation of BcII (Fig. 1*A*) but not of CphA (Fig. 1*B*). Thus, both enzymes show different affinities for zinc in the presence of substrate, and CphA is obviously able to strongly compete with EDTA for available zinc ions. When 100 μM EDTA and 90 μM Zn(II) were simultaneously present, BcII and CphA reached about 30 and 80%, respectively, of the activity measured in the presence of Zn(II) without EDTA. The free zinc ion concentration available for the reconstitution of the enzymes was in the picomolar range (see Fig. 2). With the recently determined second order rate constant for zinc binding to apo-BcII of $k_{on} = 14\ \mu M^{-1}\ s^{-1}$ (9), the half-life time for reactivation of apo-BcII via the pool of free zinc ions can be calculated ($t_{1/2} = \ln 2/(k_{on}[Zn]_{free})$) and results in, for example, 80 min for a free zinc concentration of 10 pM. Because the process is finished within a few seconds, it follows that a pool of free zinc ions cannot be relevant for reactivating the enzyme. It might be concluded that Zn-EDTA and apoenzyme form a ternary EDTA-Zn(II)-BcII complex that allows a fast transfer of zinc ions to the enzyme binding site in a ligand exchange reaction.

Whereas BcII and L1 were fully activated within the dead time of the experiments (~5 s), the reactivation of CphA is characterized by a lag phase. At first glance the lag phase might indicate a slow association of zinc ions because of its limited concentration. However, the same effect is observed with 100 nM Zn(II) in the absence of EDTA (Fig. 1*B*). The most plausible explanation for these results is a fast formation of a stable apoenzyme-substrate complex with CphA, which blocks the access of zinc ions to the metal binding site. The observed reactivation rate would thus be determined by the dissociation rate of the substrate from the apoenzyme.

On the basis of these observations we developed a method for the determination of dissociation constants for zinc ions during activity measurements in the presence of EDTA. The steady-state rates observed at different $[Zn]_{total}/[EDTA]_{total}$ ratios are a direct measure of the enzyme fractions simultaneously loaded with (one or two) zinc ions and substrates according to Equation 13. With Equations 3–12, K_{mono} and K_{bi} could be determined numerically together with the parameters for the spe-

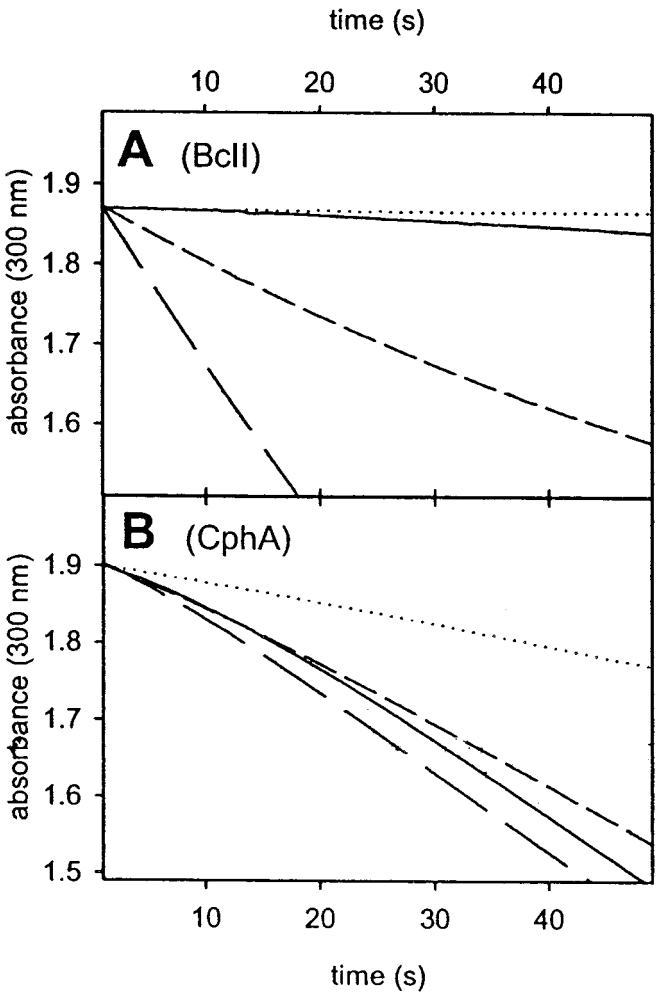

FIG. 1. **Reactivation kinetics of BcII and CphA in the absence and presence of EDTA.** Time courses of imipenem cleavage by BcII (*A*) and CphA (*B*) were monitored at 300 nm. Starting conditions for both enzymes were about 200 μM imipenem in 15 mM HEPES, pH 7.0, at 25 °C. The monitored reactions were started by the addition of apoenzymes to a final concentration of 40 and 3 nM for BcII and CphA, respectively. In the absence of EDTA in the reaction medium both enzymes showed a reactivation by incorporating zinc ions from the metal-depleted solution. This process was slow for BcII (*A*, *continuous line*) and completed within ~25 s for CphA (*B*, *continuous line*). The presence of 100 μM EDTA in the reaction medium prevented the reactivation of BcII (*A*, *dotted line*) but could only reduce the reactivation of CphA (*B*, *dotted line*). The addition of 100 μM Zn(II) to the reaction medium resulted in the spontaneous reactivation of BcII (*A*, *long dashed line*), whereas the reactivation of CphA with 100 nM Zn(II) was characterized by a lag phase of 20 s (*B*, *long dashed line*). The simultaneous presence of 90 μM zinc ions and 100 μM EDTA led to a 30% reactivation of BcII (*A*, *short dashed line*) and a 80% reactivation of CphA (*B*, *short dashed line*) when compared with experiments in the presence of Zn(II) and the absence of EDTA.

cific activity of the two different enzyme species $k_{app,ZnE}$ and k_{app,Zn_2E}. In Fig. 2 relative activities are used based on the maximum activity obtained under the chosen conditions. After the optimization of K_{mono} and K_{bi}, Equations 3–12 were used to calculate $[Zn^{2+}]_{free}$ from the experimental data and the corresponding theoretical curves (Fig. 2, *right panels*). For all enzymes investigated two binding steps were observed, one in the picomolar and one in the micromolar range, resulting in two enzyme species with different activities. The apparent dissociation constants obtained when substrates are present ($K_{mono,sub}$ and $K_{bi,sub}$ in Table I) reflect the $[Zn]_{free}$ needed to result in 50% of the enzymes acting as either Zn_1E or Zn_2E under conditions of substrate saturation and strongly deviate from those determined in the absence of substrates.

It is evident that the activities measured do not give a direct estimate of enzyme concentrations. Both $k_{app,ZnE}$ and $k_{app,Zn2E}$ are functions of the respective K_m values and the substrate concentration used. Additional inhibiting effects of EDTA can modify the activities observed. For CphA and BlaB inhibition constants of 1.75 and 1.54 mM, respectively, have been reported (4, 17). The values for $k_{app,ZnE}$ and k_{app,Zn_2E} resulting from the fits do not therefore reflect true specific activities (k_{cat}) but include the saturation state of the respective enzyme species. This becomes obvious in the case of BcII where the binuclear enzyme appears to be only slightly more active than the mononuclear enzyme (Fig. 2*A*), although the steady-state parameters determined with the mono- and binuclear enzyme indicate a 2-fold higher specific activity (k_{cat}) of the binuclear enzyme (Table I). However, the different K_m values of Zn_1- and Zn_2-BcII result in different degrees of substrate saturation for the mono- and binuclear enzyme species. Because of experimental limitations, saturating substrate concentrations could only be applied for BlaB and Zn_1-L1. Therefore, the concentrations of enzyme-substrate complexes during steady-state were usually lower than the enzyme concentration constrained during the fitting process. To investigate the influence of the degree of substrate saturation (or the exact knowledge of the constrained apoenzyme concentration) on the dissociation constants determined, data were alternatively evaluated by constraining the apoenzyme concentration to different values. Enzyme concentrations constrained to, for example, 10% of the experimental value resulted in 10-fold increased $k_{app,ZnE}$ and k_{app,Zn_2E}, whereas the optimized dissociation constants $K_{mono,sub}$ and $K_{bi,sub}$ remained unchanged.

All experiments except those with BlaB were performed under conditions where the free zinc ion concentration ($[Zn]_{free}$) was buffered by EDTA (see "Materials and Methods"). Attempts to study BlaB under similar conditions failed, because this enzyme shows a strong inhibition by EDTA, which is not adequately described by the published inhibition constant. Therefore, a modified (but less precise) experimental approach was used (Fig. 2*D*). At a low total zinc ion concentration, the EDTA concentration was varied in a narrow, close to equimolar range to achieve variations in $[Zn]_{free}$. Under these conditions errors in the fixed apoenzyme concentration have a significant influence on the optimized values for $K_{mono,sub}$ and $K_{bi,sub}$. Alternative optimizations showed that both dissociation constants are overestimated if the enzyme concentration is overestimated; nevertheless, the $K_{mono,sub}/K_{bi,sub}$ ratio remains almost constant.

In the presence of a substrate, all of the enzymes investigated show dissociation constants in the picomolar range for a first zinc ion bound, reflecting an increase of the zinc ion affinity by up to three orders of magnitude. Based on the results from the competition experiments, appropriate conditions for the determination of the kinetic parameters of mono- and binuclear enzymes could be chosen (Table I). Until now, L1 was considered to be a binuclear enzyme requiring two bound zinc ions for activity (22). Here we show that L1 is also active as the Zn_1 enzyme. Although k_{cat} for the Zn_1-L1 is a factor of 30 lower than for the Zn_2 species, its enzymatic efficiency (k_{cat}/K_m) is only a factor of 3 lower because of the considerably lower K_m.

Recent investigations of proteins involved in the regulation of zinc homeostasis suggest that free zinc ions are virtually absent in cellular environments. This similarly applies to eukaryotic cells (11) and the bacterial cytosol for which free zinc ion concentrations below 1 fM are expected (13). Because most metallo-β-lactamases are characterized by nanomolar dissoci-

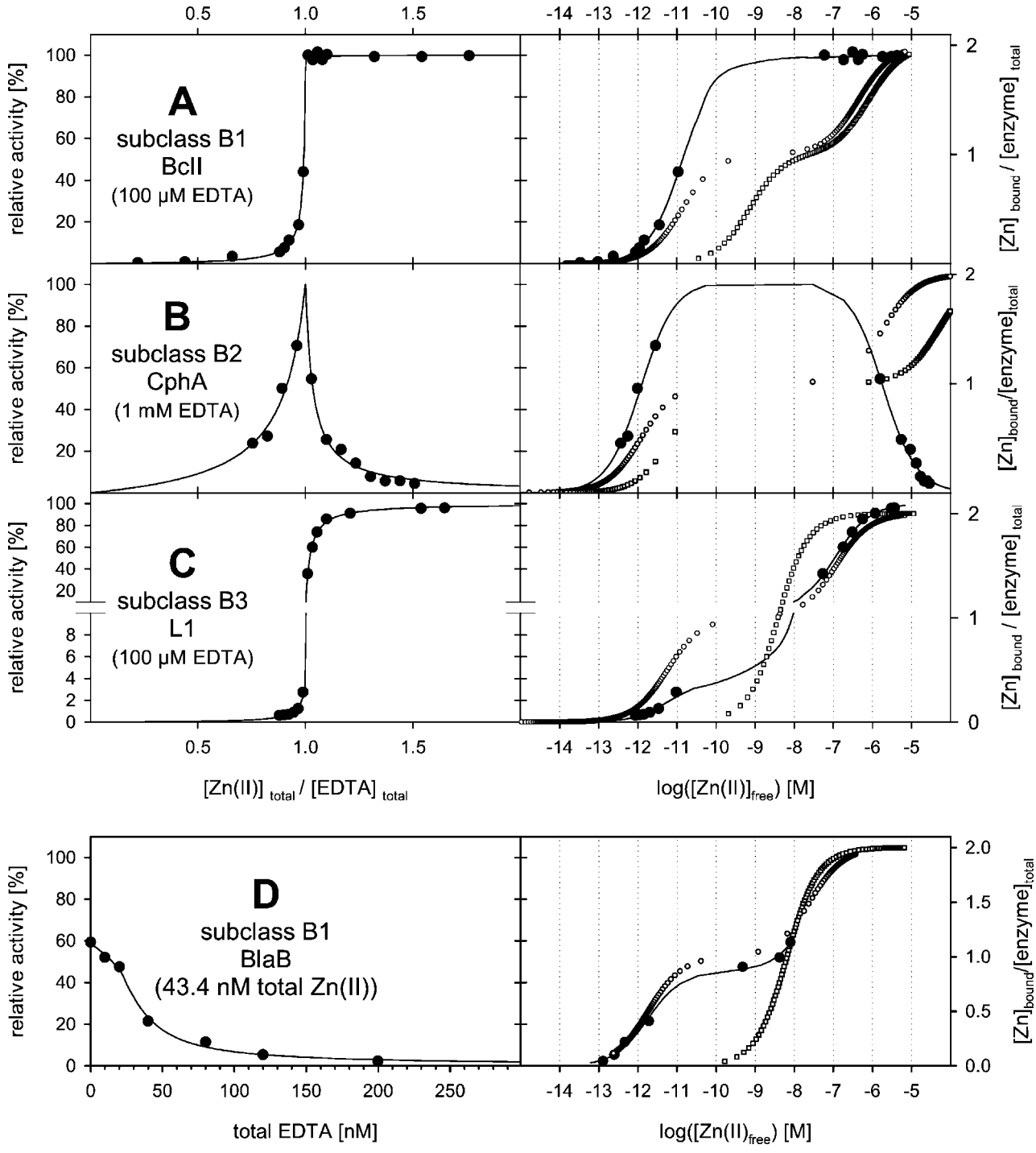

FIG. 2. **Determination of dissociation constants of Zn(II)-metallo-β-lactamase complexes in the presence of a substrate.** The competition between the different apoenzymes and EDTA was used to determine dissociation constants of the zinc enzymes in the presence of substrates as described under "Materials and Methods." Reactions were started by the addition of concentrated apoenzyme solutions to the reaction medium. Steady-state rates were determined at different $[Zn]_{tot}/[EDTA]_{tot}$ ratios and are presented as relative activities (*filled circles*) compared with rates obtained with an excess of zinc ions (*A*, *C*, and *D*) or compared with rates obtained for the Zn_1-enzyme (*B*). The averaged dissociation constants obtained from three independent experiments each are listed in Table I. The theoretical curves shown (*continuous lines*) represent simulations of the respective experiments with the best fit parameters and an increased data point density to visualize the exact shape of the theoretical curves. The respective *panels* to the *right* represent the relative activities (*filled circles*) *versus* the calculated free zinc concentration on a logarithmic scale together with the fitted theoretical curve (*continuous line*). The *open symbols* represent simulated data for the occupancy of the available enzyme with zinc ($[Zn]_{bound}/[enzyme]_{total}$, *right y*-axis). *Open circles* represent data in the presence of a substrate. For comparison, the respective saturation curves in the absence of substrates were simulated with Equations 1–3 and the dissociation constants from Table I (*open squares*). *A*, the relative activities of 92 nM BcII with a starting concentration of 0.15 mM imipenem were determined in the presence of 0.1 mM EDTA and a total zinc ion concentration ranging between 0 and 0.11 mM. *B*, the relative activities of 8 nM CphA with a starting concentration of 0.15 mM imipenem were determined in the presence of 1 mM EDTA and a total zinc ion concentration ranging between 0 and 1.1 mM. *C*, the relative activities of 26 nM L1 with a starting concentration of 0.15 mM imipenem were determined in the presence of 100 μM EDTA and a zinc ion concentration ranging between 0 and 170 μM. The *left y*-axis is split to better visualize the activities of Zn_1-L1, which are <5% of the activity of the Zn_2-enzyme. *D*, metal-free BlaB (22 nM) was incubated with 2 eq of Zn(II) (44 nM) and varied concentrations of EDTA (0–200 nM) in 30 mM sodium cacodylate, pH 6.5. The relative activities were determined with nitrocefin (100 μM starting concentration, *filled circles*) by the addition of substrate to the preincubated enzyme. The relative activities are plotted *versus* the total EDTA concentration.

ation constants in the absence of a substrate, it seems likely that these are inactive apoenzymes in such environments. Furthermore, the nanomolar zinc ion concentrations needed to constitute the active zinc enzymes were shown to be inhibitory for other enzymes like caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (23).

To our knowledge, no data concerning the free zinc ion concentration in the periplasm or the extracellular space of growing bacteria in their natural habitat are documented in the literature. Because numerous periplasmatic and extracellular enzymes are zinc proteins, a strong competition can be expected resulting in very low concentrations of free zinc ions. We have demonstrated here that the presence of substrates strongly decreases the dissociation constant of Zn(II)-metallo-β-lactamases, rendering them able to successfully compete with other zinc proteins for the available zinc ions. Thus, the presence of substrates can be considered a signal for the autoregulatory self-activation of apo-metallo-β-lactamases. Such regulation could also prevent the weak peptidase activity (k_{cat}/K_m $<0.1 M^{-1}$ s^{-1}) demonstrated for metallo-β-lactamases (24), because fully active zinc-β-lactamases could interfere with the DD-peptidase-catalyzed cross-linking of the bacterial cell wall peptidoglycan.

An important aspect of the presented results is the high reactivation rate observed in the presence of EDTA. The virtual absence of free zinc ions in cellular systems necessarily results in very long half-life times for a successful constitution of active zinc enzymes via the pool of free zinc ions. However, zinc binding is fast in biological systems, and a direct transfer via discrete (ternary) intermediates was postulated to resolve this problem (25). In the present study, EDTA serves as an artificial zinc supplier for the constitution of zinc-β-lactamases from the apoenzymes, whereas other zinc-binding factors, most likely proteins, might supply the necessary zinc ions *in vivo*.

CONCLUSIONS

For all three subclasses of metallo-β-lactamases we discovered the following functional characteristics. 1) All the enzymes studied are active as the monozinc enzyme. 2) The substrate presence generally results in a drastic increase of affinity for a first zinc ion. 3) The dissociation constant for a second zinc ion in the presence of a substrate is 4–6 orders of magnitude higher than the first, picomolar mononuclear dissociation constant.

Because of the very low concentrations of free zinc ions in cellular environments, metallo-β-lactamases are most likely in the apoenzyme state in the absence of substrates. Substrate availability induces a spontaneous self-activation already at picomolar free zinc ion concentrations, resulting in the formation of active mononuclear zinc enzymes. The binuclear state is only stabilized at unphysiologic high zinc concentrations and might therefore be considered an artificial state. The fast activation observed in competition with EDTA cannot depend on the pool of free zinc ions. A direct transfer of zinc by ligand exchange reactions in an EDTA-Zn(II)-enzyme ternary complex is postulated as the activation mechanism. *In vivo* alternative delivery systems can be suggested, such as other zinc binding proteins. It might be concluded that a direct transfer of zinc ions among binding sites is generally required to explain the fast kinetics of zinc ion transfer to newly produced binding sites.

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ENZYME CATALYSIS AND REGULATION:

Substrate-activated Zinc Binding of Metallo- β-lactamases: PHYSIOLOGICAL IMPORTANCE OF THE MONONUCLEAR ENZYMES

Sandra Wommer, Sandrine Rival, Uwe Heinz, Moreno Galleni, Jean-Marie Frère, Nicola Franceschini, Gianfranco Amicosante, Bodil Rasmussen, Rogert Bauer and Hans-Werner Adolph

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