Zinc Ion-induced Domain Organization in Metallo-β-lactamases

A FLEXIBLE “ZINC ARM” FOR RAPID METAL ION TRANSFER?\textsuperscript{1,2}

Received for publication, March 31, 2009 Published, JBC Papers in Press, April 24, 2009, DOI 10.1074/jbc.M109.001305

Nathalie Selevsek\textsuperscript{1,}, Sandrine Rival\textsuperscript{3}, Andreas Tholey\textsuperscript{1,4}, Elmar Heinzie\textsuperscript{5}, Uwe Heinz\textsuperscript{1,}, Lars Hemmingsen\textsuperscript{1,}, and Hans W. Adolph\textsuperscript{1,}\textsuperscript{11}

From the Departments of \textsuperscript{1}Biochemical Engineering and \textsuperscript{4}Biochemistry, Saarland University, 66041 Saarbrücken, Germany, the \textsuperscript{4}Institute for Experimental Medicine-Systemic Proteome Research and Bioanalytics, Christian-Albrechts Universität, 24105 Kiel, Germany, and the \textsuperscript{1}Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg C, Denmark

The reversible unfolding of metallo-β-lactamase from Chryseobacterium meningosepticum (BlaB) by guanidinium hydrochloride is best described by a three-state model including folded, intermediate, and unfolded states. The transformation of the folded apoenzyme into the intermediate state requires only very low denaturant concentrations, in contrast to the Zn\textsubscript{2}-enzyme. Similarly, circular dichroism spectra of both BlaB and metallo-β-lactamase from Bacillus cereus strain 569/H/9 (BcII) display distinct differences between metal-free and Zn\textsubscript{2}-enzymes, indicating that the zinc ions affect the folding of the proteins, giving a larger α-helix content. To identify the regions of the protein involved in this zinc ion-induced change, a hydrogen deuterium exchange study with matrix-assisted laser desorption ionization tandem time of flight mass spectrometry on metal-free and Zn\textsubscript{1-} and Zn\textsubscript{2}-BcII was carried out. The region spanning the metal binding metallo-β-lactamases (MBL) superfamily consensus sequence His-X-His-X-Asp motif and the loop connecting the N- and C-terminal domains of the protein undergoes a zinc ion-dependent structural change between intrinsically disordered and ordered states. The inherent flexibility even allows for the formation of metal ion-bridged protein-protein complexes which may account for both electrospray ionization-mass spectrometry as well as metal ion-bridged protein-protein complexes with matrix-assisted laser desorption ionization-time of flight mass spectrometry results obtained upon variation of the zinc/protein ratio and stoichiometry-dependent variations of \textsuperscript{199}Hg-perturbed angular correlation of γ-rays spectroscopic data. We suggest that this flexible “zinc arm” motif, present in all the MBL subclasses, is disordered in metal-free MBLs and may be involved in metal ion acquisition from zinc-carrying molecules different from MBL in an “activation on demand” regulation of enzyme activity.

The production of metallo-β-lactamases (MBLs)\textsuperscript{2} is one of the defense strategies of bacteria against β-lactam antibiotics.

\textsuperscript{1} This work was supported by European Union Contracts CT 98-0232 and HPRN-CT-2002-00,264, the Villum Kann Rasmussen Foundation, the Danish Instrument Centre for Cern, The Danish Natural Science Research Council, and the European Union via EURONS.

\textsuperscript{2} The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5 and Tables 1 and 2.

\textsuperscript{1} To whom correspondence should be addressed. Tel.: 45-3533-2451; E-mail: hwa@life.ku.dk.

\textsuperscript{2} The abbreviations used are: MBL, metallo-β-lactamase; MALDI-ToF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; ESI, electrospray ionization; HDX, hydrogen/deuterium exchange of amide protons; PAC, perturbed angular correlation of γ-rays spectroscopy; GdmHCl, guanidinium hydrochloride; NQI, nuclear quadrupole interaction; MES, 4-morpholineethanesulfonic acid; BisTris, 2-(bis(2-hydroxyethyl)-amino)-2-(hydroxyethyl)propane-1,3-diol; mc, main chain; sc, side chain.

MBLs hydrolyze the C-N bond of the β-lactam ring of these compounds using protein-bound zinc ions as cofactors (1). Their emergence in pathogenic bacterial strains and their broad substrate profile make them clinically important (2). Whereas the overall structure of all known MBLs is very similar (3), distinct differences in the set of protein ligands for bound zinc ions led to the classification into subclasses B1–B3 (4). Here we have studied the two subclass B1 enzymes BcII and BlaB from Bacillus cereus strain 569/H/9 and Chryseobacterium meningosepticum, respectively, which show 35.2% identical residues (5). The very similar structure of these enzymes is organized in an αββα sandwich (6, 7). The N- and C-terminal domains are connected by an external loop, and the active site is located in a long channel between the two domains. The binuclear zinc binding site is composed of a 3-His (3H) site and a Asp-Cys-His (DCH) site. Three metal ion ligands are located on the N-terminal domain and constitute the HXHXD motif, which is strictly conserved in proteins of the MBL super family (8). The three remaining metal ligands are located on the C-terminal domain of the proteins. Both for the native and for the cadmium-substituted enzyme, it has been shown that a single metal ion, when bound to BcII, appears to be distributed between the metal binding sites (9–11).

The metal ion requirement for catalytic activity of the three subclasses B1–B3 of MBLs is heavily debated. Although most crystal structures of subclass B1 enzymes show binuclear zinc sites (3), it was found that BcII from B. cereus 569/H/9, CcrA from Bacteroides fragilis, BlaB from C. meningosepticum, IMP-1 from Pseudomonas aeruginosa, and L1 from Stenotrophomonas maltophilia are both active as the mono- and di-zinc enzymes (9, 12–15). Recently a study with Co(II)-substituted BcII challenged this view in concluding that only the di-Co-enzyme might be catalytically active (16). The same authors came to the conclusion that also native BcII requires two bound zinc ions for activity (17). A very recent study on the Co(II)-substituted enzyme came to the conclusion that both the Co\textsubscript{1}- and the Co\textsubscript{2}-enzymes are catalytically active with the DCH site as the primary catalytic site (18).
Variable metal loading states of zinc proteins are attracting increasing interest in the field of cellular regulation processes being a key to the understanding of physiological functions of zinc sensors and metallothioneins as well as regulatory functions of zinc ions. The coexistence of zinc proteins in the metal-loaded and the metal-free form, however, requires the regulation of “free” zinc ion concentrations in narrow limits, with nM to pM concentrations in eucaryotes (19) or even much lower concentration in procaryotes (20). The issue, however, that zinc enzymes in their natural environment might be regulated by reversible metal ion binding is infrequently considered.

The impact of metal ion binding on structure and stability of MBL superfamily proteins has been studied in some detail. Zinc was found to be required for the folding of glyoxalase II (21) and arylsulfatases (22) into the native state. For CphA from *Aeromonas hydrophila*, differential scanning calorimetry and fluorescence spectroscopy demonstrated that zinc binding stabilizes the protein against denaturation with urea. The inactive Zn$_2^+$-CphA proved to be the most stable species (23). Crystal structures of metal-free and metal-loaded BcII revealed minor structural changes in the active site of the protein (6). $^{1}H,^{15}N$ heteronuclear single quantum coherence spectra of the backbones amides of BcII resulted in distinct signals for different metal ion/enzyme ratios which allowed discrimination of apoenzyme and metal-loaded states (24). Metal ion binding was considered to be essential for folding of L1 in vivo (25), and variable loading states were described in dependence of the bioavailability of various metal ions (26). We hypothesized earlier that metallo-$\beta$-lactamas are most likely in the metal-free apoenzyme state in the absence of substrates, which is because of the moderate affinity of the enzymes for zinc ions and the very low concentration of free zinc in cellular environments (14). We suggested that substrate availability might induce a spontaneous self-activation by direct transfer of zinc via ligand exchange reactions with delivery systems as substrate presence leads to a drastic increase of zinc affinity. The suggested self-activation mechanism, however, requires a direct interaction of the apoenzymes with other zinc carriers to allow a ligand exchange reaction to occur. Because such interactions might be considered as un Specific, it seemed reasonable to postulate a high structural flexibility to allow the transient formation of zinc-bridged complexes. To verify this prediction, we initiated an investigation on the role of zinc ions on folding and stabilization of MBLs with BlaB and BcII as test cases. Our results demonstrate that the systems, when unsaturated with metal ions, cannot be correctly described as being composed of variable fractions of the proteins in the loaded and unloaded state alone. We will present indications of the formation of labile, metal-bridged ternary complexes formed under such conditions. The latter may be considered as important intermediate states for metal ion transfer between identical or different zinc binding molecules in general.

**EXPERIMENTAL PROCEDURES**

Expression, Purification, Characterization, and Metal Depletion of Enzymes—BlaB from *C. meningosepticum* was produced and purified as described (27). The protein concentration was determined with ε$_{280\text{ nm}}$(BlaB) = 45670 M$^{-1}$ cm$^{-1}$. Metal-free BlaB (apoBlaB) 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 under stirring at 4 °C). EDTA was removed by dialysis against a 150-fold volume excess of 30 mM sodium cacodylate, 1.0 M NaCl, pH 6.5 (three changes), followed by two changes of the same buffer without NaCl. The antibiotic nitrocefin (Unipath OXoid, Basingstoke, UK) was used as a substrate for BlaB using Δε$_{456\text{ nm}}$ = 15,000 M$^{-1}$ cm$^{-1}$ following hydrolysis. To minimize zinc ion contaminations, buffer solutions were prepared in bi-distilled water and extensively stirred with Chelex 100 (Sigma).

BcII was prepared as described before (9) with some modifications. BcII was expressed from plasmid pET9a/BcII in *Escherichia coli* BL21 (DE3). Cells were grown at 37 °C in M9 minimal medium with 10 g/liter of glucose and 1 g/liter of NH$_4$Cl. Expression was induced by adding 1 mM isopropyl 1-thio-$\beta$-d-galactopyranoside at an optical density of 0.6 at 600 nm. After 16 h, cells were harvested by centrifugation, re-suspended in MES buffer (10 mM, 1 mM ZnCl$_2$, pH 6), and disrupted using a French press. Cytosolic proteins were separated from cell debris and purified chromatographically (9). Metal-free BcII (apoBcII) was prepared as described (14). Whereas BlaB was only studied as the metal-free or the fully zinc-loaded enzyme (obtained by adding excess of zinc), BcII was additionally studied when loaded with only 1 eq of metal ion. This, however, required a higher precision of the determined enzyme concentration. We used the published absorption coefficient (ε$_{280\text{ nm}}$(BcII) = 30,500 M$^{-1}$ cm$^{-1}$) (9), results from titrations of the apoenzyme with metal ions (see below), and spectrophotometric determination of the thiol group of the single cysteine residue of the protein using 5,5′-dithiobis-(2-nitrobenzoic acid) (the assay contained 0.1 M Tris/HCl, 300 μM 5,5′-dithiobis(nitrobenzoic acid), 1 mM EDTA, 1% SDS). The results from all methods agreed within 10% deviation. We decided to base our calculations on the free thiol concentration determined as the metal binding capacity depends on the presence of an intact active site. On the other hand, the method is sensitive enough to apply it to small protein concentrations and allows for routine controls (e.g. after storage, dialysis, or any other manipulation).

Circular dichroism spectra were recorded with a Jasco J740 at 20 °C. To allow direct comparison with binding experiments, the CD spectra were recorded in 5 mM HEPES, pH 7.0, at a protein concentration of 5 μM in cuvettes with a 1-mm light path.

Dissociation constants for binding of Zn$^{2+}$ to BcII were obtained from competition titrations with the chromophoric zinc chelator Mag-fura-2 (MF) (Molecular Probes, Eugene, OR). The experiments were performed in 15 mM HEPES, pH 7.0. Apparent dissociation constants for a first (K$_{\text{mono}}$) and a second metal ion bound (K$_{\text{di}}$) were obtained. A detailed description of the method can be found in de Seny et al. (10).

Equilibrium Unfolding Studies of BlaB—The enzyme (holo- and apoenzyme) was incubated in guanidinium hydrochloride (GdmHCl) solutions (0 – 6.4 M) buffered with 30 mM sodium cacodylate (CB), pH 6.5, at 4 °C. CB used for the incubation mixture was metal-free (apoenzyme) or contained 50 μM Zn(II) (holoenzyme). Emission spectra of intrinsic protein fluores-


$c$ were recorded at 25 °C on a Jobin-Yvon Spex spectropho-

The activity assay was composed of CB containing 50 μM

The GdmHCl concentration at the midpoint of each of the

concentrations ([Cm]) was assumed for both transitions (30)

in which m is the rate of change of ΔG with [D], and ΔG is the

constant under the same conditions as the samples but in the

activity assay, the GdmHCl concentration

less than 0.03 M because of dilution.

Analysis of the unfolding/folding was performed according to

either a two-state (28) or a three-state model (29). A linear

dependence of the free energy of unfolding on the GdmHCl

concentrations ([D]) was assumed for both transitions (30)

to the empirical relationship (31) ΔG = ΔG' - m[D], in which m is the rate of change of ΔG with [D], and ΔG' is the

standard free energy of denaturation in the absence of de-

The GdmHCl concentration at the midpoint of each of the

unfolding transitions, Cm, was determined from ΔG'/m.

In a two-state model, the signal observed at any denaturant

centration is given by

\[ \Delta S_{obs} = \frac{\Delta S_N e^{-\Delta G_N/m[D]} + \Delta S_U e^{-\Delta G_U/m[D]}}{1 + e^{-\Delta G_U/m[D]}} \]  

where R is the gas constant, T is the absolute temperature, and

ΔS_N and ΔS_U are the signals for the native and unfolded protein,

respectively.

In a three-state model the observed signal results from

\[ \Delta S_{obs} = \frac{\Delta S_N e^{-\Delta G_N/m[D]} + \Delta S_{Np} e^{-\Delta G_{Np}/m[D]} + \Delta S_U e^{-\Delta G_U/m[D]}}{1 + e^{-\Delta G_U/m[D]}} \]  

where ΔS_{Np} and ΔS_U are the signals for the native, intermedi-

eate, and unfolded protein, respectively. ΔG_{Np} and ΔG_U are the free

energies for the N to I and I to U transitions, respectively. ΔS_N and

ΔS_U were obtained with the linear extrapolation method of San-

toro and Bolen (28) and kept fixed in the three-state model fit.

Enzymatic activity (% of the reference) and center of fluorescence

(Σ(A FI)/ΣF I, calculated from the fluorescence intensities F I at

wavelength λ, with step size 0.1 nm) were fitted to Equations 1 and

2. The intercepts and the slopes of the pre- and post-unfolding

regimes were taken from fits of a two-state model to the data

(Equation 1) and were kept fixed in the three-state model fit (Equa-

tion 2). A significant red shift after incubation of the apoenzymes

for 24 h at 4 °C in the absence of GdmHCl (open circle in Fig. 1B)

resulted in a large uncertainty about the pre-unfolding regime

ΔS_N. Therefore, we decided to use the value obtained for the fresh,

non-incubated apoenzyme to determine ΔS_N in the fit.  

ESI-MS Study of Zinc Binding to BcII—Sample preparation,
equipment, and methods used have been described before (32).  
Relative appearances of different species (apo, Zn₁, Zn₂ species)
were calculated from deconvoluted spectra taking the different
charge states into account. Percentages given are based on the
assumption that all the different species can be detected with
the same probability.

Pepsin Digestion and Assignment of BcII Peptides by MALDI-

ToF/ToF MS—15 μM apoenzyme in 15 mM HEPES, pH 7 (10

μl), was diluted 1:11 with 0.1% trifluoroacetic acid solution to
decrease the pH to 2.3. Protein digestion was performed by

adding 30 μl of pepsin bead slurry (Pierce) (washed 4 times with

540 ml of 0.1% trifluoroacetic acid at 4 °C before use) and in-
cubated on ice for 10 min with occasional mixing. The resulting

peptides were separated from the pepsin beads by centrifuga-

tion at 4 °C. 10 μl of peptide mixture were loaded on a Zip-

TipC18 (Millipore Corp., Billerica, MA) for desalting, and the

peptides were eluted with 1 μl of α-cyano-4-hydroxycinnamic
acid (CHCA) matrix solution (5 mg/ml CHCA in acetonitrile/

ethanol/ trifluoroacetic acid 20/80/0.1) onto the MALDI plate

and dried under a stream of compressed air. We used a 4800

MALDI ToF/TOF™ Mass analyzer (Applied Biosystems,

Darmstadt, Germany) equipped with a 200-Hz Nd:YAG-Laser

(A = 355 nm, 3–7-ns pulse width). MS data were acquired in the

positive ion reflectron mode with 470-ns delayed extraction,

accumulating 500 laser shots using the 4000 Series Explorer™

Remote Access Client software (Version 3.5.1). Tandem mass

spectrometry (post-source decay with post-acceleration) was

performed for the sequencing of all detected peptic peptides;

no additional collision gas was used. For MS/MS measurements,

the acceleration voltage was 8 kV, and 4000 laser shots were

accumulated for each MS/MS spectrum.

Hydrogen Deuterium Exchange (HDX)—For HDX experi-

ments, 1 μl of 150 μM apoBcII or metal-substituted BcII stock

solution was incubated 1:10 with deuterated buffer (D₂O, 15

mM HEPES, pH 7.4) at 22 °C. Deuterium labeling times were

between 50 and 5900 s. Each exchange reaction was stopped by

the addition of 100 μl of 0.1% trifluoroacetic acid on ice,
decreasing the pH to 2.3. The digestion with pepsin and the

analysis of resulting peptides were performed as described

above for the non-deuterated digest. After spotting the deu-

terated samples on the MALDI target, each exchange experiment

was immediately measured by MALDI-MS with less than a

1-min delay. All solutions and ZipTips were kept cold before

use. The MALDI plate was kept at room temperature to prevent

condensation of water on the plate. All experiments were

repeated in triplicate.

The centroid mass of each isotope cluster was calculated

using the MagTran software (33) by labeling the left side of the

lowest deuterated peak and the right side of the highest deu-

terated peak. The percentage of deuterium in-exchange of amide

groups (%D) at the end of the incubation time in D₂O was deter-

mined using

\[ %D = 100 \left( \frac{m_i - m_{0%}}{m_{100%} - m_{0%}} \right) \]  

where
Structural Zinc Switch of MBLs

where \(m_i\) is the observed centroid mass of the deuterated peptide for each in-exchange time, and \(m_0\%\) corresponds to the non-deuterated mass of the corresponding peptide. Fully deuterated sample \(m_{100\%}\) was prepared by incubating 10 \(\mu\)l of peptin-digested apoBcII (15 \(\mu\)M) in deuterated buffer (D\(_2\)O, 15 mM HEPES, pH 7.4) for 72 h at 22 °C. During sample preparation and transfer to the MALDI target, back exchange of incorporated deuterium atoms to hydrogen cannot be completely suppressed. Therefore, it is essential to correct the experimental data for this back exchange by using the experimentally obtained centroid mass of fully deuterated peptides \((m_{100\%})\) after back exchange as the 100% value. The specific back exchange of each peptide was determined separately.

For data representation the experimentally obtained values were further processed. From the experimentally determined masses and was trapped in 150 mM BisTris, pH 6.8, and the protein. Finally, sucrose was added to produce a 60% w/w solution.

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

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

(Eq. 5)

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 mercury nucleus. 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). Each NQI was modeled by using a separate set of the parameters, \(v_Q, \eta, \delta, 1/\tau_c, \) and \(A\). The parameter \(v_Q\) \((v_Q = eQV_{zz}/h,\) in which \(Q\) is the nuclear electric quadrupole moment, and \(V_{zz}\) is the numerically largest eigenvalue of the electric field gradient tensor) is associated with the strength of the interaction between the surrounding electronic environment and the mercury nucleus; \(\eta\) is the so-called asymmetry parameter, which is 0 in an axially symmetric complex and has a maximal value of 1; \(\delta\) is the relative frequency spread; \(\tau_c\) is the rotational correlation time; \(A\) is the amplitude of the signal (35). 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 (36).

RESULTS

Equilibrium Unfolding/Refolding Studies of BlaB—The reversible unfolding of BlaB in GdmHCl was followed by fluorescence spectroscopic measurements. Both apo- and Zn\(_2\)-BlaB show emission maxima at 337 nm (Fig. 1A). The fluorescence intensity, however, is 30% lower for the apoenzyme, indicative of stronger quenching of tryptophan fluorescence. The emission maximum after 24 h of storage at 4 °C in the absence of GdmHCl was unchanged for the holoenzyme but red-shifted to 343 nm for the apoenzyme (data not shown), which indicates partial unfolding and, thus, a lower stability of the protein in absence of bound zinc ions. Increasing GdmHCl caused significant changes in both the emission intensity and the red shift of the emission maximum, which occurred in two stages. In the range 1.3–2.0 M for Zn\(_2\)-BlaB and 0.5–1.3 M for apoBlaB, the fluorescence intensity decreased to limiting values of 37 and 58% at 337 nm, respectively, and the emission maximum was red-shifted from 337 to 358 nm for both. A further increase of GdmHCl concentrations resulted in a re-increase of fluorescence intensity for both species, resulting in identical final spectra. Thus, the center of fluorescence may be used as an indicator of the degree of unfolding at various GdmHCl concentrations. Significant spectral changes occur with transitions at GdmHCl concentrations ~1.3 and ~0.5 M for Zn\(_2\)- and apoBlaB, respectively, indicating a higher susceptibility of the latter to the denaturing agent. In Fig. 1, B and C, the spectral changes with changing GdmHCl concentrations are compared with the enzyme activities obtained under the respective conditions when diluting the preincubated enzyme samples into a reactivation buffer before starting the activity assay. Whereas the fluorescence data represent the unfolding process measured in the presence of GdmHCl, the activity data represent the refolding process measured in buffer containing almost no GdmHCl. The activity data indicate the presence of a non-reactivating intermediate. Whereas the unfolded state \(U\) of apoBlaB can undergo 100% refolding to the native state (Fig. 1B), the refolding of the \(U\) state of Zn\(_2\)-BlaB only results in an ~30% refraction (Fig. 1C). Obviously the presence of zinc during the unfolding process influences the refolding step, although the reaction buffer for both apo- and Zn\(_2\)-BlaB contains 50 \(\mu\)M added zinc. To further explore the Zn\(_{2+}\) dependence, we repeated the refactoring experiments of Zn\(_2\)- and apoBlaB preincubated at 6.4 M GdmHCl with zinc concentrations between 50 and 320 \(\mu\)M in the reaction buffer. No influence of increasing zinc concentrations was observed for Zn\(_2\)-BlaB, but the reactivated fraction obtained for apoBcII dropped from initially >80% to a value below 40%, confirming that the degree of refolding does indeed depend on the Zn\(_{2+}\) concentration. The GdmHCl concentrations yielding the lowest activity (Fig. 1, B and C) and fluoro-
thermodynamic analysis of the unfolding data required a three-
state model of folded, partially unfolded, and unfolded states
(Equation 2). The corresponding fits to fluorescence and activity
data are shown in Fig. 1, A and B. The resulting parameters are combined in Table 1. The total stability of the folded state is given by the sum of $\Delta G^0$ values for both steps, resulting in $7 \pm 1$ and $14.6 \pm 0.5$ kcal/mol for apo- and Zn$_2$-BlaB, respectively.

Nano-ESI-MS Investigation of Putative Zinc Loading States of BcII at Variable Zinc/Protein Stoichiometries—ESI-MS spectra of apoBcII under denaturing conditions (methanol, 0.2% formic acid) showed typical high charge distribution states (+16 and +32) at low m/z values (m/z 800 and 1600) (data not shown), whereas low charge distribution states (+8 and +10) at high m/z values (m/z 2500 and 3100) were measured under “nondenaturing” conditions. The calculated mass from these signals was in agreement with that predicted from the gene sequence of BcII, and Zn$_2$-BcII (24,960 Da). A systematic variation of $[\text{Zn}^{2+}]$ was in agreement with that predicted from the gene sequence of BcII, and Zn$_2$-BcII (supplemental Fig. 1). These results indicate that the zinc enzyme required 24 h of incubation with GdmHCl to fully observe the spectroscopic changes, whereas the apoenzyme did show 100% of changes already right after mixing with the denaturing agent. The thermodynamic analysis of the unfolding data required a three-state model of folded, partially unfolded, and unfolded states (Equation 2). The corresponding fits to fluorescence and activity data are shown in Fig. 1, A and B, and the resulting parameters are combined in Table 1. The total stability of the folded state is given by the sum of $\Delta G^0$ values for both steps, resulting in $7 \pm 1$ and $14.6 \pm 0.5$ kcal/mol for apo- and Zn$_2$-BlaB, respectively.

Under nondenaturing conditions (NH$_4$HCO$_3$, pH 7), the assignment of the different metal-loaded species resulted from subtracting the observed mass of apoenzyme from the mass of metal-loaded species (supplemental Fig. 1). For zinc titrations, typical masses at 25,018 $\pm$ 0.18 Da and 25,082 $\pm$ 0.26 Da were observed corresponding to Zn$_1$-BcII and Zn$_2$-BcII species (loss of two protons for each bound Zn(II) with an average mass of 65.3 Da). Surprisingly, all spectra of BcII obtained at $[\text{Zn}^{2+}] < 2$BcII indicated the simultaneous presence of apoBcII, Zn$_1$-BcII, and Zn$_2$-BcII (supplemental Fig. 1). These results indicate positive cooperativity of zinc ion binding to BcII and are in clear contradiction to our earlier published data (10, 14).

This prompted us to repeat the corresponding competition experiments with Mag-Fura-2 (supplemental Fig. 2), resulting in $K_{\text{mono}} = 0.12$ nM and $K_{\text{stoichiometric}} = 0.99$ nM. The titrations gave no indication of positive cooperativity for zinc ion binding. Data
**Structural Zinc Switch of MBLs**

### TABLE 1

**Fit results for the three-state equilibrium unfolding of BlaB in GdmHCl**

The thermodynamic parameters $\Delta G^0$ (kcal/mol), $\Delta G^0_{H2O}$, and the characteristics of the intermediate state, $\Delta S$, obtained from the fits in Fig. 1, B and C, to activity and fluorescence data are summarized. The parameters obtained for the three-state model were used to calculate midpoint concentrations of GdmHCl ($C_m$) for the transitions $N$ to $I$ and $I$ to $U$.

| Activity | $\Delta S$ | $\Delta G^0$ | $m_N$ | $\Delta G^0_{H2O}$ | $m_I$ |
|----------|-------------|--------------|--------|---------------------|--------|
| Apo      | 13%         | 2.2 ± 0.4    | 5.0 ± 0.9 | 5.5 ± 1.0          | 2.4 ± 0.4 |
| Zinc     | 0%          | 9.8 ± 0.5    | 7.1 ± 0.4 | 4.5 ± 0.7          | 2.0 ± 0.3 |

### TABLE 2

**Relative abundance of signals for apo-$\zeta$, Zn$_1$-, and Zn$_2$-BcII in ESI-MS spectra upon variation of Zn$^2+$/protein**

ApoBcII at a concentration of 11.7 $\mu$M was incubated with the given zinc ion concentrations. Equation 6 was used to calculate the ratio of apparent dissociation constants for a first and second zinc ion bound ($K_{mono}/K_{bi}$) in a sequential binding model.

| $\text{Zn}^{2+}$ total | Relative peak intensities | $K_{mono}/K_{bi}$ |
|------------------------|--------------------------|-------------------|
| $\mu$M                 | ApoBcII | Zn$_1$-BcII | Zn$_2$-BcII | ApoBcII | Zn$_1$-BcII | Zn$_2$-BcII |
| 5                      | 61.5    | 18          | 20.5        | 3.9     |
| 10                     | 39      | 19          | 42          | 4.5     |
| 12                     | 24      | 22          | 54          | 2.7     |
| 14                     | 9.5     | 14.5        | 76          | 3.5     |
| 30                     | 100     |             |             |         |

---

simulation was extensively used to investigate whether any combination of $K_{mono}$ and $K_{bi}$ with $K_{bi} < K_{mono}$ might be able to fit the titration data. This turned out to be impossible. Thus, there appears to be a contradiction between the MS and competition experiment data obtained for the same enzyme batch.

It has to be pointed out that the new $K_{bi}$ is significantly lower than earlier published values (10, 14). A possible explanation for the deviations in the equilibrium constants results from different preparation protocols. Whereas the enzyme for the present study was obtained from E. coli BL21 (DE3), cells grown in M9 minimal medium, earlier studies were performed with BcII derived from the same E. coli strain but grown in LB medium (37). BcII preparations obtained from LB medium-grown E. coli were shown by ESI-MS to be composed of an ensemble of protein fractions with ragged N termini (38) and additionally contained a pigment of unknown chemical nature that was co-purified with the enzyme (37). Neither peculiarities were observed in the minimal medium-derived preparation of BcII used in this work, whereas in some of our earlier studies (9, 10, 14, 39) LB medium-derived BcII was used. SDS-PAGE of the purified enzyme resulted in a single band (supplemental Fig. 3), and neither a previously published ESI-MS study of the BcII batch used here (32) nor the mass spectrometric data shown here for the intact protein (ESI-MS) and the peptide fragments obtained by pepsin digestion (MALDI-ToF/ToF MS) gave any indication of heterogeneity of the protein sample in terms of variable total mass or any kind of chemical modification.

**Circular Dichroism Spectroscopy at Variable Zinc/Protein Stoichiometries**—To probe structural differences, we performed CD spectroscopic measurements with apoBcII samples with 1 or 2 eq of zinc added in comparison to data obtained for BlaB (Fig. 2). The Zn$_2$-enzyme spectra show strongly increased intensities of the negative band at 220 nm as compared with the metal-free enzymes and indicate an increased $\alpha$-helix content of the Zn$_2$ species. The spectroscopic differences between apoenzyme and fully zinc-loaded species are very similar for BcII and BlaB. Whereas the intensity of the 220 nm band is identical for Zn$_1$- and Zn$_2$-BcII, an additional shoulder at 210 nm appears in the spectrum of the Zn$_2$-enzyme.

**Selection of Peptidic Peptides for HDX Studies Based on Assignment Using MALDI-ToF-ToF MS**—The structural view of hydrogen/deuterium exchange kinetics requires the fragmentation of a protein and the separate investigation of smaller peptide fragments. The experimental protocol was standardized with respect to preparation time and temperature before MALDI measurement. Peptide mass fingerprint analysis of pepsin-digested apoBcII resulted in the identification and assignment of 22 peptides in the mass range of $m/z$ 800–3500 (supplemental Fig. 4 and supplemental Table 1). The average mass accuracy obtained for these peptides was 3.5 ppm. Examples for experimental data are presented in supplemental Fig. 4. The average back exchange for all
peptic peptides was ~40%, but individual values for each peptide were determined and used for data correction. All experiments were performed in triplicate starting from the sample preparation. Depending on the total number of exchanged amide protons in a peptide, the resulting standard deviations are highly variable, ranging from 2 to 12%. In supplemental Table 1 all the peptides included in the study are listed. HDX after 2000 s is compared for apo-, Zn1-, and Zn2-BcII. The total number of exchangeable amide protons is compared with the experimentally observed exchange. Some sequence sections are found in several peptides due to alternative cleavage sites of pepsin. We selected 14 peptides with 96% sequence coverage and mapped them on the Zn2-BcII structure in Fig. 3. The three metal ion ligands of the C-terminal domain, namely His-149, Cys-168, and His-210, are found on the peptide fragments P-(139–155), P-(165–188), and P-(205–219), respectively. The typical MBL superfAMILY sequence motif HXXH is found on P-(82–110) from the N-terminal domain. In the following analysis, only these 14 peptides are used.

FIGURE 3. HDX kinetics of BcII. The time courses of percent deuterium in-exchange for various parts of the protein are shown on a logarithmic time scale. Experimental data for the different enzyme species are presented as follows: circles, apoBcII; half-filled circles, Zn1-BcII; filled circles, Zn2-BcII. The theoretical curves are represented as lines through the data points and were obtained from fitting Equation 4 to data (results are summarized in supplemental Table 2). The percentage of amide hydrogens not involved in main chain-main chain hydrogen bonds is indicated by full horizontal lines, and the percentage of amide hydrogens involved in neither main chain-main chain nor main chain-side chain hydrogen bonds is represented by broken horizontal lines. The latter data were obtained from an inspection of the crystal structure of Zn2-BcII (PDB code 1bvt).
Structural Zinc Switch of MBLs

Quantification and Structural Interpretation of HDX Kinetics—A combined representation of kinetic HDX data is shown in Fig. 3. The total time courses consist of a very rapid phase, completed within the dead time of our experiments, and a time-resolved phase which is best described by a monoexponential function (Equation 4).

The inspection of the structure of native Zn2-BcII (PDB accession code 1bvt) reveals a correlation of the amplitudes of the rapid HDX phase and the percentage of amide protons not involved in hydrogen bonds. Three types of amide protons are classified as follows: (i) amide protons involved in main-chain-main chain (mc-mc) hydrogen bonds. They comprise spatially neighboring peptide bonds found in α-helices, β-sheets, or turns (126 in total). They are shielded against solvent access and, thus, show generally slow HDX. (ii) The same might hold for amide protons involved in hydrogen bonds to side chains and, thus, show generally slow HDX. (iii) Best solvent access and highest rates of HDX are expected if all the amide hydrogens not involved in hydrogen bonds were exchanged (horizontal lines). For fully metal-loaded species, the percentage of non-hydrogen-bonded amide protons correlates well with the first data points obtained (compare Fig. 3). Thus, it might be concluded that at least all the hydrogen-bonded amide protons are largely protected against HDX for incubation times <50 s. The fitted deuterium exchange during the dead time of our experiments, thus, mainly covers non-hydrogen-bonded amide protons. In turn, enzyme species showing a considerably higher percentage of HDX at t = 50 s compared with the fully metal-loaded species might have structures with a decreased number of hydrogen-bonded amide protons within the respective peptide segments.

In general, apoBcII displays a higher HDX within the experimental dead time (%D0) compared with the metal-loaded samples, and this is particularly prominent for three peptide segments involved in the structural organization of the metal binding site, namely P-(82–110), P-(82–110), and P-(115–129). This indicates that the amide protons do not participate to hydrogen bonding to the same extent in the apoprotein as in the fully zinc-loaded species and, thus, that the secondary structure elements are not as well defined in these fragments of the apoenzyme.

199mHg-PAC Spectroscopy of BcII at Different Metal/Protein Stoichiometries—Aiming at a detection of metal ion-bridged protein dimers of BcII, we studied binding of Hg2+ to BcII. The reasons for using Hg2+ are described under “Discussion.” Spectra were recorded at the ISOLDE facility at CERN (Switzerland). Hg2+ binding to apoBcII was studied by adding 1 (Fig. 4A) and 0.1 eq of Hg2+ (Fig. 4B) to 30 μM apoBcII in 100 mM BisTris, pH 6.8, containing 60% sucrose. The spectrum obtained with 1 Hg2+ added per protein molecule could be fitted with a single nuclear quadrupole interaction (NQI 1: \( \nu_0 = 1.09 \pm 0.01 \text{ GHz}, \) \( \eta = 0.296 \pm 0.016, \) \( 1/\tau_c = 27 \pm 9 \text{ μs}^{-1}, \) \( \delta = 0, \) \( A = 0.113, \)) reflecting one dominant coordination geometry. The spectrum obtained with only 0.1 eq of Hg2+ added contained statistically significant contributions of a second NQI. The fit resulted in NQI 1 (\( \nu_0 = 1.08 \pm 0.01 \text{ GHz}, \) \( \eta = 0.293 \pm 0.025, \) \( \delta = 0, \) \( A = 0.088 \)) and NQI 2 (\( \nu_0 = 1.50 \pm 0.03 \text{ GHz}, \) \( \eta = 0.215 \pm 0.061, \) \( \delta = 0, \) \( A = 0.042 \)), contributing 68 and 32% to the total amplitude, respectively. The rotational correlation time resulted as an average for both NQIs in \( 1/\tau_c = 2 \pm 11 \text{ μs}^{-1}. \) One of these signals is very similar to that observed with 1 eq of Hg2+, and the other falls in the range observed for dihedral HgS2 coordination in model systems (34, 40, 41).

DISCUSSION

The present investigation aims to describe MBLs and their metal ion binding capacity in terms of flexibility and dynamics. Our starting hypothesis that MBLs may be highly dynamic proteins resulted from a combination of various observations previously described in the literature. A rapid transfer of zinc ions from strong binding carriers like EDTA to the proteins has been shown to require a direct interaction of MBLs with the zinc carrier (14). The latter, however, presupposes a considerable flexibility of the metal binding site to accommodate a zinc loaded EDTA molecule. In another study, a rapid change of the coordination geometry of MBL-bound cadmium ions was described. Hemmingsen et al. (24) could show that a single bound Cd2+ ion apparently jumped between the two metal sites of BcII in a time regime between 0.1 and 10 μs. This finding is...
Global Effects of Bound Zinc on Structure and Stability of MBLs—In comparison to Zn\(_2\)-BlaB, the apoenzyme shows less intense tryptophan fluorescence at 337 nm (Fig. 1A) and a decreased intensity of the negative 220-nm band in the CD spectrum (Fig. 2B). Both findings indicate structural differences between the two enzyme states, resulting in stronger fluorescence quenching of one or more tryptophan residues and a potential decrease in e.g. \(\alpha\)-helix content for the apoenzyme. Circular dichroism spectra (Fig. 2A) of BcII show very similar results. Thus, zinc binding obviously influences the structure of the proteins. That zinc binding increases the stability against unfolding with chaotropic reagents like GdmHCl is apparent from Fig. 1. Whereas Zn\(_2\)-BlaB Resists up to 1 M GdmHCl without significant changes of its fluorescence spectrum and without any change of its activity (Fig. 1C), the apoenzyme shows significantly decreased activity under refolding conditions and a significant shift of the center of fluorescence already with 0.2 M GdmHCl (Fig. 1B). The theoretical description of the denaturation with GdmHCl requires a three-state unfolding model involving an intermediate state (I) in addition to the native (N) and the unfolded (U) state. The intermediate state is characterized by a minimum in the activity versus GdmHCl curves (Figs. 1, B and C) and a state of lowest fluorescence intensity (Fig. 1A). The thermodynamic parameters obtained by fitting Equation 2 to the activity and the fluorescence data are very similar, and average values are used for the following argumentation. Zinc binding stabilizes the enzyme by 8.9 ± 1.2 kcal/mol. The difference in thermodynamic stability exclusively originates from the N to I transition, which is characterized by \(\Delta G^0\) of only 1.7 ± 0.5 kcal/mol for the apoenzyme. This corresponds to an equilibrium constant of \(K = |I|/|U| = 0.056\). At 3 \(\mu\)M total protein (compare Fig. 1) and 25 °C, this results in ~5% of the apoenzyme residing in the I state. This observation might also explain the low stability of the apoenzyme upon storage and the need to use a fresh apoenzyme sample for the GdmHCl = 0 M value in Fig. 1B. Thus, the apoenzyme, which is partly unfolded, as implied by the fluorescence and CD spectra, may bear pronounced characteristics of the intermediate state. To localize the parts of the MBL structure involved in the stabilization by zinc ion binding, we initiated a HDX study on BcII.

Local Effects of Bound Zinc on Structure and Stability of MBLs; Zinc-induced Ordering of a Domain—It has been shown earlier that hydrogen/deuterium exchange studied by mass spectrometry (HDX-MS) may be used to monitor metal ion binding-induced conformational changes of metalloproteins because such changes are coupled to a modified total number of amide protons protected against HDX (42, 43). Only few investigations, however, used HDX-MS combined with proteolysis of metalloproteins to identify the individual protein regions affected by metal ion binding (44, 45). Here we compare the structural flexibility of metal-free BcII and the enzyme loaded with one or two zinc ions.

In our study the absence of bound metal ions increased the solvent accessibility of a number of regions in the protein; highly buried parts close to the active site as well as regions far from the active site. The kinetic analysis results in the highest in-exchange during the experimental dead time (%D\(_0\)) and/or time-resolved in-exchange (%D\(_i\)) for the apoenzyme. For all the
peptides the percentage of un-exchanged amide protons at the end of the time-resolved phase ($\%H_{\text{end}} = \Sigma (\text{amide protons}) - \%D_0 - \%D_1$) is lowest for apoBcII.

Few parts of the structure are almost unaffected by metal ion binding. This is most striking for P-(70–81), which does not show any statistically relevant differences between the kinetic parameters for the different species. This peptide fragment may, therefore, be considered as an “internal standard” for validating deviations in kinetic parameters between different species observed for other fragments. The most pronounced effects of metal ion binding are observed for three peptides from the N-terminal domain, namely P-(82–110), P-(115–129), and P-(82–110) (see Fig. 3).

In the crystal structure P-(82–110) comprises $\alpha$-helix II spanning residues 88–101 and the MBL superfamilies consensus sequence HXHXXD motif, which makes up half of the metal binding ligands for the binuclear zinc site. In $\text{Zn}_2$-BcII (PDB code 1bvt) 20 of 29 amide protons from P-(82–110) are involved in 16 mc-mc and 4 mc-sc interactions. Consequently the in-exchange during the dead time ($\%D_0$) for $\text{Zn}_2$-BcII is below 30% (compare supplemental Table 2). For apoBcII, however, only 6 of the 29 NH protons in total are un-exchanged after 50 s of incubation in D_2O, which reflects an almost unstructured state of this peptide segment. This means that the secondary structure elements, including $\alpha$-helix II, are most likely unstructured in apoBcII. The two other peptide fragments, which show similar effects of metal ion loading, namely the peptide connecting the N- and C-terminal domains P-(115–129) and P-(82–110), are hydrogen-bonded to P-(82–110) in the crystal structure of $\text{Zn}_n$-BcII. The connecting loop P-(115–129) is bound to P-(82–110) via one mc-mc and three mc-sc hydrogen bonds. P-(82–110) is bound to P-(82–110) via three mc-mc and three sc-sc hydrogen bonds including salt bridges of the guanidinium group of Arg-91 to Asp-90 and Asp-56. Apparently the destabilization of the P-(82–110) segment resulting from the absence of bound metal ions induces a coupled destabilization of the sequence segments covered by P-(82–110) and P-(115–129) due to the lack of structure-stabilizing hydrogen bonds between the segments. Thus, the segment spanning residues 82–129 appears to be at least transiently unstructured in apoBcII. The CD results also indicate a reduced $\alpha$-helix content in apoBcII, further supporting the interpretation of the MS data. We conclude that zinc binding is required to stabilize the structure of the motif depicted in Fig. 5C, which harbors peptides P-(82–110) and P-(115–129). We suggest that this motif may undergo zinc ion-induced ordering. A structural alignment of MBLs from all three subclasses shows that the overall structure of this domain is highly conserved (Fig. 5C), indicating that the flexibility of the domain is a common feature of all MBLs.

To further explore the flexibility of the domain spanning residues 82–129 from a theoretical perspective, we applied DisProt VSL2B (46, 47), RONN (48), and DisEMBL (49) as predictors for intrinsic protein disorder in BcII and BlaB. All three programs resulted in qualitatively very similar results in predicting at least parts of the zinc switch domain as intrinsically disordered regions. It has to be emphasized that all these predictors do not consider structure-stabilizing effects of bound metal ions and, thus, reflect the properties of the apoproteins. That is, the theoretical predictions agree with the flexibility of the zinc switch domain observed experimentally for the apoproteins. With few exceptions, the regions predicted by VSL2B to have a high probability of being disordered also show a high HDX within the dead time of our experiments (supplemental Fig. 5). Predictions for the sequences of BcII and BlaB show very similar results. Interestingly, the sequence segment P-(165–188) harboring the single cysteine residue, which is a metal ion ligand in the DCH site, is also predicted to be intrinsically disordered and shows high HDX, which is, however, almost independent of the metal loading state (Fig. 3).

$\text{Zn}_2^+$- versus $\text{Zn}_2^-$-BcII; Metal Ion-bridged Species May Account for Apparently Contradicting Results—The preceding comparisons intentionally did not discriminate the different loading states of BcII with zinc and mainly compared $\text{Zn}_2^-$-enzyme and apoenzyme. In the following we intend to highlight the peculiar characteristics of BcII in case only one zinc ion is available per enzyme molecule. To date all descriptions of the MBL-metal system are based on the assumption that single protein molecules are either existent as metal-free units or units loaded with one or two metal ions. In the following we will demonstrate that this perception is inadequate to explain the apparently contradicting results obtained with different methods and should be reframed by additionally considering metal ion-bridged complexes of two or more MBL molecules. Surprisingly, all ESI-MS spectra of BcII obtained at $\text{Zn}^{2+} < 2\text{BcII}$ indicated the simultaneous presence of apoBcII, $\text{Zn}_1$-BcII, and $\text{Zn}_2$-BcII (Table 2). Even without added metal the residual zinc in the apoBcII preparation was obviously sufficient to allow detection of the $\text{Zn}_n$ species (supplemental Fig. 1). Earlier published dissociation constants for BcII (10, 14) resulted in a nanomolar and a micromolar $K_d$ for a first and second zinc ion bound, respectively. Thus, one would have expected to observe only apoBcII and $\text{Zn}_2$-BcII up to $\text{Zn}^{2+}$-BcII = 1. The most obvious explanation for detection of $\text{Zn}_n$-species at low $\text{Zn}^{2+}$/protein requires that $K_{\text{bi}} < K_{\text{mono}}$, which means positive cooperativity of zinc ion binding. Because of the fact that ESI-MS data neither allow the quantification of free zinc nor give direct access to absolute concentrations of the different enzyme species, it is not possible to use the data for direct determinations of dissociation constants. Whereas the absolute values for both $K_d$ values of a sequential binding model cannot be obtained, the apparent ratio of the two constants can be calculated from the relative abundances of metal-free and metal-loaded enzyme species according to Equation 6 (see Table 2).

$$\frac{K_{\text{mono}}}{K_{\text{bi}}} = \frac{[\text{Me}_2\text{E}]^2}{[\text{MeE}]^2}$$

(Eq. 6)

Although MS is not yet a fully validated method in this context, an average ratio $K_{\text{mono}}/K_{\text{bi}} = 3.7$ for zinc ion binding to BcII indicates positive cooperativity. Competition experiments with Mag-Fura-2, however, delivered $K_{\text{mono}}/K_{\text{bi}} = 0.12$. Thus, the ESI-MS study and the competition titration gave contradicting results in that they appear to reflect positive cooperativity of zinc ion binding or not, respectively.
An additional apparent discrepancy appears when comparing CD and MS data. The CD spectra with 1 and 2 eq of Zn(II) added to the apoenzyme (Fig. 2A) are very similar with an additional shoulder at 210 nm in the spectrum of the Zn₂-enzyme. Under the assumption that the ESI-MS data obtained reflected the species distribution in solution, it is straightforward to derive 37.27, 25.47, and 37.27% for apo, Zn₁-, and Zn₂-BcII, respectively, for the experimental conditions used in the CD experiment. This calculation is based on the average Kₐ/Kₜₐₙₙ of 3.7 (Table 2). A hypothetical CD spectrum for pure Zn₁-BcII (see Fig. 2A) can then be calculated according to (Equation 7).

\[
\text{CD}_{Zn₁,\text{calc}} = 3.93(\text{CD}_{Zn₁} - 0.37\text{CD}_{apo} - 0.37\text{CD}_{Zn₂})
\]  

(Eq. 7)

Large deviations from both apoenzyme and Zn₂-BcII are obvious. Because of the spectral characteristics, only significantly increased α-helix content could account for the observed deviations. This, however, would mean that Zn₁- and Zn₂-BcII should have significantly different folds which should be reflected in the results obtained from the comparative HDX investigation on the three different enzyme species.

For apoBcII, the three peptides P-(82–110), P-(82–110), and P-(115–129) show increased in-exchange during the dead time (%Dₐ) compared with the Zn₂ species (Fig. 3). The amplitudes of the time-resolved phases for these peptides are quite small, and the number of finally un-exchanged amide protons is 90 for Zn₁-BcII, which is only slightly less than found for Zn₂-BcII. Although the three peptides show increased %Dₐ values for Zn₁-BcII, others fall below the values found for Zn₂-BcII, namely the four peptides from the C-terminal domain spanning residues 156–219. This is most pronounced for P-(205–219), which comprises the metal ion ligand His-210 and appears to be best protected against HDX in Zn₁-BcII. Especially the latter results are difficult to understand when assuming only monomeric states of the protein as it appears counterintuitive to assume that the Zn₁ state could experience a stronger stabilization compared with the Zn₂ state. The latter findings, however, might be explained from the formation of BcII-Zn₂-BcII complexes by stabilization of protein segments upon interaction with the neighboring molecule. Attempts to directly detect dimers of Zn-loaded BcII by ESI-MS upon variation of the experimental conditions (data not shown) failed, however, possibly because of insufficient stabilities to survive the ionization process.

Aiming at the detection of possibly existing dimers of BcII, we studied binding of Hg²⁺ to BcII with ¹⁹⁹mHg PAC spectroscopy. Considering the high flexibility observed in the HDX study and the apparent lack of defined interactions between N- and C-terminal domains, it appeared possible to create new metal ion binding sites by combining residues from two protein molecules. The idea was that the very strong affinity of Hg²⁺ for sulfur ligands and its preference for formation of dihedral S-Hg-S complexes might result in the formation of complexes with Hg²⁺ bridging the single Cys residues of two BcII molecules. It has been shown earlier that such dihedral S-Hg-S coordination can be clearly detected by ¹⁹⁹mHg PAC spectroscopy (34, 50). We compared two different stoichiometries, namely 1 and 0.1 eq of Hg(II) added to apoBcII. The resulting spectra (Fig. 4) showed clear differences. Whereas the Hg₀-BcII spectrum could be fitted with a single nuclear quadrupole interaction (NQI 1: \(\nu_Q = 1.09 \pm 0.01\) GHz and \(\eta = 0.296 \pm 0.016\), the Hg₀₁-BcII spectrum could not. NQI 1 could also be identified in the Hg₀₂-BcII spectrum, but additionally an NQI 2 with \(\nu_Q = 1.50 \pm 0.03\) GHz and \(\eta = 0.215 \pm 0.061\) was necessary to obtain a satisfactory fit. The contributions to the total amplitude are 68 and 32% for NQI 1 and NQI 2, respectively. At present, further investigations are under way to obtain a structural assignment of NQI 1. A comparison with literature data, however, results in the assignment of NQI 2, which is only seen at low stoichiometry as being due to a dihedral S-Hg-S coordination slightly deviating from a linear arrangement (34, 40, 41). The formation of such a complex, however, requires the direct interaction of two BcII molecules via a bridging Hg²⁺ as only one Cys is available per protein molecule. Thus, the ¹⁹⁹mHg PAC data indicate that metal-free BcII is flexible enough to allow the formation of such complexes, and we hypothesize that similar complexes might also be formed at substoichiometric availability of zinc ions.

The fact that we could not find a coherent interpretation of all the experimental data with a binding model only covering three states of BcII, namely apoBcII, Zn₁-BcII, and Zn₂-BcII, required extension of the binding model to include additional states of the protein. The HDX study resulted in a proven flexibility of the apoenzyme and the lack of structural organization with respect to the domain-domain interaction and organization of the metal binding site. Additionally HDX demonstrated that some parts of the protein are better stabilized with only 1 eq of zinc bound; e.g. P-(205–219) shows the strongest stabilization against HDX in Zn₁-BcII, which might be easily explained from protein-protein interactions. This prompted us to hypothesize that metal ion-bridged complexes might exist in which e.g. two BcII molecules supply ligands for the same metal ion(s). The ¹⁹⁹mHg PAC studies evidenced that such complexes can be formed at least with mercury as the bridging metal ion.

In the following we will demonstrate how the involvement of such complexes, e.g. BcII-Zn₁-BcII, might abrogate the apparently contradicting results. The metal ion binding sites of such dimers might be composed such that e.g. the HXHXD motif is contributed by protein molecule A and the C-terminal metal ion ligands by molecule B. This model agrees well with the CD data if the secondary structure elements are already stabilized by the presence of only one zinc ion per enzyme molecule and at the same time accounts for the fact that the spectra of the mono- and di-zinc species display minor but distinct differences.

The competition titrations (supplemental Fig. 2) show that a first zinc equivalent binds stronger compared with a second zinc equivalent bound. The simple binding model (only including apoBcII, Zn₁-BcII, Zn₂-BcII) is fully sufficient for the theoretical description as the method is not able to discriminate the formation of Zn₁-BcII from formation of BcII-Zn₂-BcII. The only apparently contradicting result remaining at this stage is the observation of Zn₂-BcII by ESI-MS upon sub-stoichiometric addition of zinc (supple-
Structural Zinc Switch of MBLs

mental Fig. 1). When assuming that the ESI-MS data exactly reflected the situation in solution before the ionization process took place, the results clearly indicate positive cooperativity of zinc ion binding to BcII. When assuming, however, that complexes like BcII-Zn₂-BcII exist in solution and that these complexes are to labile to survive the ionization process, they might statistically decompose either into two Zn₁-BcII complexes or into one molecule of apoBcII and one Zn₂-BcII complex upon ionization. Thus, the previous existence of monomeric Zn₂-BcII in solution is not necessarily required to explain its detection in the ESI-MS spectra. If the Zn/BcII system has a strong tendency to form BcII-Zn₂-BcII dimers at low availability of zinc ions, this could also explain why Zn₂-BcII can even be detected in apoBcII samples with only residual zinc available for binding (supplemental Fig. 1).

Functional Implications and Conclusions—In an earlier study it was hypothesized that MBLs in the absence of substrates might exist as the metal-free apoenzymes in their native bacterial environment. There the free zinc ion concentration is too low to maintain a metal-loaded state when considering the moderately high affinities of MBLs reported (14). The appearance of substrates in the periplasm of bacteria, which is the native site of action of MBLs, would then require the loading of apoMBLs with zinc to establish activity. Substrates have been shown to increase the affinity of MBLs for a first zinc ion bound to picomolar dissociation constants, whereas a second zinc ion is bound by 3–4 orders of magnitude weaker under such conditions (14). For L1 from S. maltophilia it has been shown that the second metal ion bound might get lost during substrate turnover (Ref. 3 and references therein). Thus, the Zn₁ state of MBLs might be the functional state in vivo.

If free zinc is virtually absent, however, only a direct transfer between zinc-loaded competitors and apoMBLs via intermolecular ligand exchange could allow for a rapid activation in the presence of substrates. For such intermolecular transfer mechanisms, the flexibility of apoBcII observed here may be a prerequisite that the transient formation of complexes with competing zinc carriers appears more probable than would be the case with a preformed metal binding site in a deep groove of the protein structure. Such a mechanism has been suggested for zinc transfer between MBLs and EDTA (14). It has been shown earlier for zinc finger peptides that substoichiometric availability of metal ions might lead to the formation of metal ion bridged complexes (51). Thus, we propose the ability of MBLs to form such complexes if the metal ion binding site concentration exceeds the concentration of available metal ions. If the transient formation of metal ion-bridged protein-protein complexes is part of the activation mechanism of MBLs at low zinc ion concentrations, the presence of substrate might be required to stabilize the active state of the proteins (14) and trap the metal ion(s) in the active site as activation on demand. If the Zn₁ state is considered the functional state of MBLs under in vivo conditions (3, 14), the dynamics observed here might also be important for catalysis. In a present freeze-quench PAC study on Cd(II)-substituted BcII we try to investigate metal binding dynamics during substrate turnover. This method allows following substrate-induced coordination geometry changes of the metal ion on a millisecond time scale. Whether the structural zinc switch domain identified in this work has similar functions in other MBL superfamily proteins remains to be seen.

Acknowledgments—We thank Wolfgang Maret for discussions and Marianne Lund Jensen for technical assistance during PAC experiments and acknowledge ISOLDE/CERN for beam time.

REFERENCES

1. Frère, J. M. (1995) Mol. Microbiol. 16, 385–395
2. Walsh, T. R., Tolemann, M. A., Poirel, L., and Nordmann, P. (2005) Clin. Microbiol. Rev. 18, 306–325
3. Heinz, U., and Adolph, H. W. (2004) Cell. Mol. Life Sci. 61, 2827–2839
4. Gallen, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O., and Frère, J. M. (2001) Antimicrob. Agents Chemother. 45, 660–663
5. Rossolini, G. M., Franceschini, N., Riccio, M. L., Mercuri, P. S., Perilli, M., Gallen, M., Frère, J. M., and Amicosante, G. (1998) Biochem. J. 332, 145–152
6. Carfi, A., Duée, E., Gallen, M., Frère, J. M., and Dideberg, O. (1998) Acta Crystallogr. B. 54, 313–323
7. García-Saez, I., Hopkins, J., Papamicael, C., Franceschini, N., Amicosante, G., Rossolini, G. M., Gallen, M., Frère, J. M., and Dideberg, O. (2003) J. Biol. Chem. 278, 23868–23873
8. Aravid, I. (1999) In Silico Biol. 1, 69–91
9. Paul-Soto, R., Bauer, R., Frère, J. M., Gallen, M., Meyer-Klaucke, W., Nolting, H., Rossolini, G. M., de Seny, D., Hernandez-Valladares, M., Zeppzejauer, M., and Adolph, H. W. (1999) J. Biol. Chem. 274, 13242–13249
10. de Seny, D., Heinz, U., Wommers, S., Kiefer, M., Meyer-Klaucke, W., Gallen, M., Frère, J. M., Bauer, R., and Adolph, H. W. (2001) J. Biol. Chem. 276, 45065–45078
11. Paul-Soto, R., Zeppzejauer, M., Adolph, H. W., Gallen, M., Frere, J. M., Carfi, A., Dideberg, O., Wouters, J., Hemmingen, L., and Bauer, R. (1999) Biochemistry 38, 16500–16506
12. Costello, A. L., Sharma, N. P., Yang, K. W., Crowder, M. W., and Tierney, D. L. (2006) Biochemistry 45, 13650–13658
13. Paul-Soto, R., Hernandez-Valladares, M., Gallen, M., Bauer, R., Zeppzejauer, M., Frère, J. M., and Adolph, H. W. (1998) FEBS Lett. 438, 137–140
14. Wommers, S., Rival, S., Heinz, U., Gallen, M., Frere, J. M., Franceschini, N., Amicosante, G., Rasmussen, B., Bauer, R., and Adolph, H. W. (2002) J. Biol. Chem. 277, 24142–24147
15. Siemann, S., Breuer, D., Clarke, A. J., Dmitrienko, G. I., Lajoie, G., and Viswanathan, T. (2002) Biochim. Biophys. Acta 1571, 190–200
16. Badarau, A., and Page, M. I. (2006) Biochemistry 45, 11012–11020
17. Badarau, A., and Page, M. I. (2008) J. Biol. Inorg. Chem. 13, 919–928
18. Larrull, L. I., Tioni, M. F., and Vila, A. J. (2008) J. Am. Chem. Soc. 130, 15842–15851
19. Krezel, A., and Maret, W. (2007) J. Am. Chem. Soc. 129, 10911–10921
20. Outten, C. E., and O’Halloran, T. V. (2001) Science 292, 2488–2492
21. Dragani, B., Cocco, R., Ridderström, M., Stenberg, G., Mannervik, B., and Aceto, A. (1999) J. Mol. Biol. 291, 481–490
22. Melino, S., Capo, C., Dragani, B., Aceto, A., and Petruzzelli, R. (1998) Trends Biochem. Sci. 23, 381–382
23. Hernandez, V. M., Felici, A., Weber, G., Adolph, H. W., Zeppzejauer, M., Rossolini, G. M., Amicosante, G., Frère, J. M., and Gallen. M. (1997) Biochemistry 36, 11534–11541
24. Hemmingen, L., Damblon, C., Antony, J., Jensen, M., Adolph, H. W., Wommers, S., Roberts, G. C., and Bauer, R. (2001) J. Am. Chem. Soc. 123, 10329–10335
25. Periyannaan, G., Shaw, P. J., Sigdel, T., and Crowder, M. W. (2004) Protein Sci. 13, 2236–2243
26. Hu, Z., Gunasekera, T. S., Spadafora, L., Bennett, B., and Crowder, M. W. (2008) Biochemistry 47, 7947–7953
27. Vessillier, S., Docquier, J. D., Rival, S., Frere, J. M., Gallen, M., Amicosante, A.
G., Rossolini, G. M., and Franceschini, N. (2002) Antimicrob. Agents Chemother. 46, 1921–1927
28. Santoro, M. M., and Bolen, D. W. (1988) Biochemistry 27, 8063–8068
29. Nath, U., and Udgaonkar, J. B. (1995) Biochemistry 34, 1702–1713
30. Schellman, J. A. (1978) Biopolymers 17, 1305–1322
31. Pace, C. N. (1986) Methods Enzymol. 131, 266–280
32. Selevsek, N., Tholey, A., Heinzle, E., Liénard, B. M., Oldham, N. J., Schofield, C. J., Heinz, U., Adolph, H. W., and Frère, J. M. (2006) J. Am. Soc. Mass Spectrom. 17, 1000–1004
33. Zhang, Z., and Marshall, A. G. (1998) J. Am. Soc. Mass Spectrom. 9, 225–233
34. Iranzo, O., Thulstrup, P. W., Ryu, S. B., Hemmingsen, L., and Pecoraro, V. L. (2007) Chemistry 13, 9178–9190
35. Hemmingsen, L., Sas, K. N., and Danielsen, E. (2004) Chem. Rev. 104, 4027–4062
36. Hemmingsen, L., Bauer, R., Bjerrum, M. I., Adolph, H. W., Zeppezauer, M., and Cedergren-Zeppezauer, E. (1996) Eur. J. Biochem. 241, 546–551
37. de Seny, D., Prosperi-Meys, C., Bebrone, C., Rossolini, G. M., Page, M. L., Noel, P., Frère, J. M., and Galleni, M. (2002) Biochem. J. 363, 687–696
38. Payne, D. J., Skett, P. W., Aplin, R. T., Robinson, C. V., and Knowles, D. J. (1994) Biol. Mass Spectrom. 23, 159–164
39. Heinz, U., Bauer, R., Wommer, S., Meyer-Klaucke, W., Papamichals, C., Bateson, J., and Adolph, H. W. (2003) J. Biol. Chem. 278, 20659–20666
40. Butz, T., Volkel, T., and Nuyken, O. (1991) Chem. Phys. 149, 437–443
41. Faller, P., Cortecka, B., Tröger, W., Butz, T., and Vasák, M. (2000) J. Biol. Inorg. Chem. 5, 393–401
42. van den Bremer, E. T., Jiskoot, W., James, R., Moore, G. R., Kleanthous, C., Heck, A. J., and Maier, C. S. (2002) Protein Sci. 11, 1738–1752
43. Nemirovskiy, O., Giblin, D. E., and Gross, M. L. (1999) J. Am. Soc. Mass Spectrom. 10, 711–718
44. Wang, F., Li, W., Emmett, M. R., Marshall, A. G., Corson, D., and Sykes, B. D. (1999) J. Am. Soc. Mass Spectrom. 10, 703–710
45. Gonzalez de Peredo, A., Saint-Pierre, C., Latour, J. M., Michaud-Soret, I., and Forest, E. (2001) J. Mol. Biol. 310, 83–91
46. Peng, K., Radivojac, P., Vucetic, S., Dunker, A. K., and Obradovic, Z. (2006) BMC Bioinformatics 7, 208
47. Obradovic, Z., Peng, K., Vucetic, S., Radivojac, P., and Dunker, A. K. (2005) Proteins 61, Suppl. 7, 176–182
48. Yang, Z. R., Thomson, R., McNeil, P., and Esnouf, R. M. (2005) Bioinformatics 21, 3369–3376
49. Linding, R., Jensen, L. J., Diella, F., Bork, P., Gibson, T. J., and Russell, R. B. (2003) Structure 11, 1453–1459
50. Troger, W., and Butz, T. (2000) Hyperfine Interact. 129, 511–527
51. Heinz, U., Kiefer, M., Tholey, A., and Adolph, H. W. (2005) J. Biol. Chem. 280, 3197–3207