Spectroscopic Characterization of a Binuclear Metal Site in *Bacillus cereus* β-Lactamase II†

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ABSTRACT: The zinc metalloenzyme β-lactamase II (βLII) from *Bacillus cereus* has been overexpressed in *Escherichia coli* as a fusion protein with glutathione-*S*-transferase, and the metal binding properties of recombinant βLII toward Zn(II) and Co(II) have been studied by fluorescence and activity measurements. The apoenzyme is able to bind two metal ion equivalents, which confer on βLII its maximum enzymatic efficiency. The enzyme is partially active with one metal ion equivalent. The diCo(II) and a mixed Zn(II)Co(II) derivative of βLII were obtained and probed by electronic and paramagnetic NMR spectroscopy. In the high-affinity site, the metal is bound to three His residues and a solvent molecule, adopting a tetrahedral geometry. A Cys, a His, and an Asp residue are coordinated to the low-affinity metal site, together with two or three solvent molecules. This coordination polyhedron resembles the binuclear metal site of the *Bacteroides fragilis* β-lactamase [Concha, N., Rasmussen, B. A., Bush, K., and Herzberg, O. (1996) *Structure 4*, 823−836; Carfi, A., Duée, E., Paul-Soto, R., Galleni, M., Frère, J. M., and Dideberg, O. (1998) *Acta Crystallogr. D54*, 47−57] but differs from that resulting from the X-ray study of βLII [Carfi, A., Pares, S., Duée, E., Galleni, M., Duez, C., Frère, J. M., and Dideberg, O. (1995) *EMBO J. 14*, 4914−4921]. These results suggest that this binuclear metal site may be a general feature of metallo-β-lactamases.

β-lactamases are bacterial enzymes that have developed the ability to hydrolyze the β-lactam ring of several antibiotics. Most of the bacterial resistance to antibiotics arises from the production of these enzymes. Prototypical β-lactamases are serine-active enzymes, classified into different categories having distinct hydrolytic mechanisms as well as different substrate specificities *(1−3)*. The so-called class B β-lactamases are an exception, since their activity depends on the presence of a divalent metal ion, naturally zinc(II). Metallo-β-lactamases represent only 7% of the universe of β-lactamases, but they stand as the major group of carbapenem-hydrolyzing enzymes *(4)*. The interest on expanding the current knowledge on these enzymes has been stimulated by the diffusion of metallo-β-lactamases to pathogenic species and the inefficacy of commercial inhibitors against their hydrolytic action *(4, 5)*. A thorough understanding of the metallo-β-lactamases' structure and the mechanism of the reactions they catalyze may provide the background for rational drug design.

β-lactamase II (βLII)[1] from *Bacillus cereus* was the first enzyme of this class to be reported *(6, 7)*. Early NMR experiments were useful in defining the involvement of His residues in the metal binding site *(8, 9)*. Substitution of the native Zn(II) by Co(II) gave rise to a characteristic Cys → Co(II) LMCT at 348 nm, thus indicating a probable His_3Cys coordination polyhedron *(7, 10, 11)*. The recently solved X-ray structure of βLII revealed an active site containing one Zn(II) ion coordinated to three histidine residues (His 86, His 88, and His 149) and to a solvent molecule in a tetrahedral fashion *(12)* (Figure 1A), in conflict with the spectroscopic studies in Co(II)-substituted βLII *(7, 10, 11)*. This discrepancy could be attributed in principle to differences in the coordination spheres between the Zn(II) and Co(II) enzymes. However, the subsequent elucidation of the 3D structure of the metal-dependent β-lactamase from *Bacteroides fragilis* provided more intriguing clues to this subject *(13−15)*. This enzyme possesses an active site with two Zn(II) ions *(13−16)*: one of them (Zn_1) exhibits a coordination polyhedron superimposable with the only Zn(II) ion found in βLII (Figure 1B). The second one (Zn_2) displays a trigonal bipyramidal coordination geometry, with a solvent molecule as bridging ligand between the two ions (Figure 1B). Zn_2 is also coordinated to an Asp, a Cys, a His, and an additional solvent molecule. Since all the Zn_2

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[1] Abbreviations: βLII, β-lactamase II from *Bacillus cereus*; CDNB, 1-chloro-2,4-dinitrobenzene; ε, extinction coefficient; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GST, glutathione-*S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; LMCT, ligand-to-metal charge transfer; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WEFT, water-enhanced Fourier transform.

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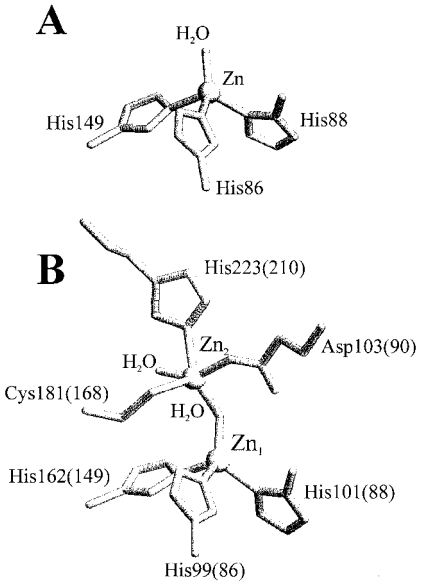

FIGURE 1: Schematic view of the metal sites of (A) βLII, from Carfi et al. *(12)*; and (B) *Bacteroides fragilis* β-lactamase, from Concha et al. *(13)*. The pictures were rendered with RasMol v2.6 (R. Sayle, 1994, Greenford, Middlesex, U.K.). The amino acid numbering given in parentheses for the picture of the *B. fragilis* enzyme indicate the corresponding numbering in βLII.

ligands are conserved residues in βLII *(17)*, and previous binding studies support the existence of a second metal site in this enzyme *(7, 10)*, the finding of only one metal ion in βLII has been attributed to a lower metal affinity. One of the major issues stemming from these data is whether these enzymes indeed display different catalytic mechanisms or not.

Metal substitution is a well-established protocol for probing metal sites in proteins *(18−24)*, being particularly useful in zinc enzymes since the Zn(II) ion is silent to most spectroscopic techniques. This strategy has also been applied to study βLII *(7, 10, 11)* and the *B. fragilis* β-lactamase *(15, 16)*. In the former case, the discrepancies with the X-ray structure have cast some doubt on the validity of extending the spectroscopic data on the Co(II) derivative to the native enzyme. However, Co(II) has been recognized as a superb probe since Co(II)-substituted zinc enzymes retain mostly the native active-site geometry and activity *(25−30)*. We have studied the binding of both Zn(II) and Co(II) to apo-βLII by using different spectroscopic techniques. Due to the large quantities of enzyme needed for these studies, we have overexpressed βLII in *Escherichia coli* as a fusion protein with GST.

In the present paper we will address the following issues: How many metal sites are there for Zn(II) and Co(II) binding in βLII? Do the conserved amino acids in the putative second metal-binding site of βLII lead to a binuclear center similar to the one in the *B. fragilis* enzyme? Are there substantial differences between the Zn(II) and Co(II) derivatives of this enzyme? How does the occupancy of different metal sites correlate to enzymatic efficiency?

EXPERIMENTAL PROCEDURES

All chemicals used in this work were purchased commercially and were of the highest quality available. The MTPBS buffer is 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 150 mM NaCl, pH 7.3. The Tris-Zn buffer is 100 mM Tris-HCl and 1 mM $ZnSO_4$, pH 7.0.

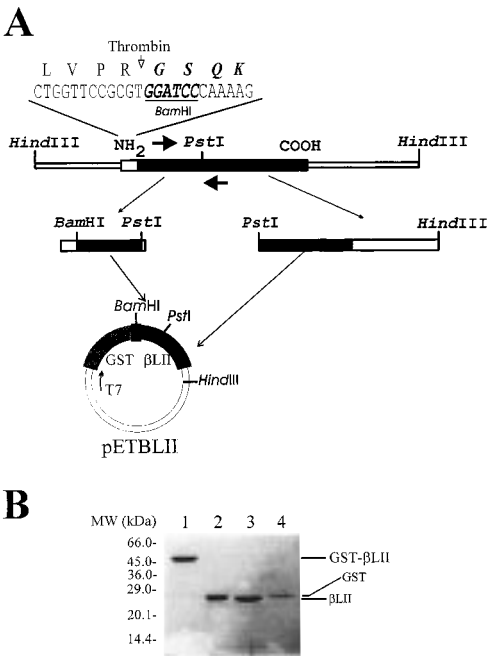

FIGURE 2: Vector construction and expression of the GST−βLII fusion protein. (A) Construction of the pETBLII vector. The sequence at the top indicates the oligonucleotide used to introduce a *Bam*HI site and a sequence codifying a thrombin recognition cleavage site (indicated with an arrow). The solid areas within recombinant plasmids and in spare fragments represent DNA coding for βLII; the stippled areas, cDNA of GST; and the open areas, plasmid DNA. (B) Electrophoretic analysis of the proteins: 4 μg of purified GST−βLII fusion protein (lane 1), 4 μg of the proteolysis mixture (lane 2), 5 μg of purified βLII (lane 3), and 1.5 μg of purified GST (lane 4) were subjected to SDS−12% PAGE followed by staining with Coomassie Brilliant Blue. The positions corresponding to the molecular weight markers are indicated at the left margin. The positions of the protein bands are indicated at the right margin.

Plasmid Construction. cDNA clones for βLII from *B. cereus* 569/H (pRWH012) *(17)* were kindly provided by Dr. A. Carlino (Roche). The recombinant plasmid pETBLII (Figure 2A) contains the entire sequence of mature βLII linked in frame to the 3′-end of *Schistosoma japonicus* GST gene. This plasmid was constructed as follows: a 522 bp fragment containing the NH_2-terminal region of βLII was amplified by using the following primers: 5′-CTGTACAA-GGATCCCAAAAGGTAGAGA-3′ and 5′-TTCACTAAA-CAGCCTCCAACTAAAAT-3′. The amplified fragment contains a *Bam*HI restriction site as well as a sequence encoding a cleavage recognition site of thrombin (Figure 2A, top). This fragment was digested with *Bam*HI and *Pst*I. The product of 336 bp was purified and ligated with a 1050 bp *Pst*I−*Hind*III fragment containing the COOH-terminal fragment of the original βLII cDNA and introduced in *Bam*HI−*Hind*III sites of a pETGEX-CT vector *(31, 32)* (Figure 2A). The sequence coding for the recognition site of thrombin is then located immediately 5′ to the cloning site of βLII. This construction was verified by DNA sequencing.

Protein Expression and Purification. βLII from *B. cereus* 569/H was obtained as described by Davies et al. *(33)*. Recombinant βLII from *E. coli* was obtained as follows: BL21 (DE3) *E. coli* cells transformed with the plasmid pETBLII were grown in 13-L cultures in a Microferm unit

(New Brunswick) from an overnight inoculum, in LB medium supplemented with 150 μg/mL ampicillin. Cells were grown 4−5 h at 37 °C until $OD_{600} = 0.8$ was reached. IPTG was added, and the culture was further incubated at 30 °C for an additional 2 h period. All subsequent purification steps were performed at 4 °C. *E. coli* cells were collected by centrifugation at 8000*g* for 5 min and then resuspended in lysis buffer (MTPBS with 1 mM $ZnSO_4$ and 1 mM PMSF) and lysed in a French press at 1200 psi. Cell debris were separated after ultracentrifugation at 105000*g* for 1 h. DNase (2 μg/mL) was added to the clear supernatant liquid, which was loaded into a glutathione−agarose resin (sulfur linkage, Sigma) equilibrated with MTPBS buffer. The solution was passed several times overnight in order to ensure maximum binding of the fusion protein. The column was thoroughly washed with MPTBS buffer and 50 mM Tris-HCl buffer at pH 8. The GST−βLII fusion protein was eluted with 10 mM GSH in 50 mM Tris-HCl at pH 8. The pure fractions were treated with bovine plasma thrombin (Sigma) with a 8:500 ratio in 150 mM NaCl/2.5 mM $CaCl_2$ at 26 °C during 1 h under gentle agitation. The proteolysis mixture was diluted to 1:3 with buffer and later passed through a Sephadex CM-50 column equilibrated with Tris-Zn buffer. The resin was washed until no absorption at 280 nm and no GST activity were detected in the eluate. Pure βLII was finally eluted with Tris-Zn buffer containing 400 mM NaCl.

Activity and Protein Measurements. β-Lactamase activity was monitored by using cephalosporin C, penicillin G, or nitrocefin as substrates. The reaction medium was 50 mM MOPS, pH 7; 20 mM sodium succinate/1 M NaCl, pH 6.0; or 20 mM sodium cacodylate/0.5 M NaCl, pH 6.0. In each case, the final concentration of Zn(II) was 10 mM, except during the titrations. The kinetic parameters were determined with nitrocefin as substrate by following the product formation by the increase of Abs_{482} ($\Delta\epsilon_{482} = 15\,000\ M^{-1}\ cm^{-1}$) *(34)* by initial rate measurements. One unit of β-lactamase activity is defined as the amount that hydrolyzes 1 μmol/min of substrate/min at 30 °C in 20 mM sodium cacodylate/0.5 M NaCl, pH 6.0, in the presence of 10 mM $ZnCl_2$. In the titrations followed by activity measurements, the apoenzyme was added to the reaction mixture containing the substrate and the metal ion at the corresponding concentration. Preincubation of the apoenzyme with the metal ion, followed by addition to the reaction mixture containing the substrate yielded comparable values. In all cases, the stability of the substrate in the presence of Zn(II) or Co(II) in the corresponding buffer was tested before each assay.

GST activity was monitored at 30 °C by using the transferase-mediated reaction between GSH and CDNB *(35)*. The reaction course was followed by the increase of Abs_{340} in a mixture containing 1 mM GSH and 1 mM CDNB in 100 mM sodium phosphate buffer at pH 6.5.

Total protein concentrations were estimated by Lowry's method *(36)*. Protein samples were analyzed by SDS−PAGE followed by Coomassie blue staining or by inmunoblotting. Samples were denatured by incubation in 10 mM Tris-HCl, pH 7, 2 mM EDTA, 0.5% β-mercaptoethanol, 10% (v/v) glycerol, and 2% SDS for 5 min in a boiling water bath and then separated by SDS−PAGE in a 12% gel *(37)*. After electrophoresis the samples were transferred to nitrocellulose membrane and inmunoblot analysis were carried out using rabbit antisera raised against GST and βLII *(38)*. N-Terminal protein sequencing was performed by the Protein/Peptide Micro Analytical Laboratory at the California Institute of Technology (Pasadena, CA).

Metal Derivatives. The apoprotein was prepared by dialyzing a 0.1 mM solution of βLII twice against 100 volumes of 10 mM EDTA in 20 mM sodium succinate/1 M, pH 6 at 4 °C; and then against at least two times with 100 volumes of 20 mM sodium succinate/1 M NaCl, pH 6 at 4 °C *(8, 9)*. All these procedures were performed in Chelex-treated solutions. Plasticware was washed with Chelex-treated water prior to use. The integrity of the apoprotein was checked in all cases by the recovery of the β-lactamase activity upon addition of 10 mM Zn(II). The Co(II) derivative was prepared by addition of cobalt chloride to a buffered solution of apoprotein at room temperature. A standard cobalt solution (Titrisol, Merck) was used for this purpose.

Spectroscopic Measurements. All spectra were collected at 25 °C, except when indicated. The electronic spectra were recorded in Gilford Response II, Beckman DU 620, and Ultraspec II LKB spectrometers. Spectrophotometric titrations were performed by stepwise addition of standard solutions of Co(II) and Zn(II) chloride to a 10 mm path length cuvette containing 0.2−8 mM apo-βLII in metal-free buffer. After a 5-min equilibration, spectra were recorded. The read value remained unchanged after the equilibration time. The reported spectra were obtained by subtracting the spectrum of the starting apoenzyme or the excess Co(II) (when appropriate). In all cases, the spectra were corrected for dilution. The protein concentration in the cuvette was determined from $\epsilon_{280} = 22\,000\ M^{-1}\ cm^{-1}$.

Metal binding was followed by fluorescence measurements using a Jasco FP-700 fluorometer. Tryptophan fluorescence was recorded with the excitation wavelength at 286 nm (10-nm slit) and emission at 335 nm (10-nm slit). In a typical experiment, apo-βLII was added to a final concentration of 0.2−2 μM to 2−3 mL of a metal-free buffer in the fluorometer cuvette. Aliquots of a metal ion solution were added, and after a 5-min equilibration, the fluorescence was read. Usually, 8−10 data points spanning the sensitive range of the binding curve(s) were collected. The fraction of formed Co(II)-βLII was determined by the fluorescence drop at each point. The measured fluorescence data were corrected for dilution and inner filter effects. EDTA was added after the titrations in order to check the reversibility of metal binding and the absence of protein denaturation during the experiment. The measured fluorescence after addition of EDTA usually amounted to 80−90% of the value corresponding to the apoenzyme. The corrected fluorescence data were fit to a hyperbolic equation using a nonlinear least-squares regression.

NMR spectra were recorded on Bruker ACE 200 and MSL 300 spectrometers operating at proton frequencies of 200.13 and 300.13 MHz, respectively. The concentrated samples for NMR experiments were obtained by using Centricon-10 (Amicon) concentrator units up to final concentrations of 2−5 mM. D_2O solutions were prepared by dissolving the lyophilized protein in deuterium oxide. The reported pH values measured in D_2O solutions are not corrected for the isotopic effect. All chemical shifts were referenced to the chemical shift of water at the appropriate temperature, which

Table 1: Kinetic Parameters Determined for Nitrocefin Hydrolysis

	B. cereus βLII	recombinant βLII	GST−βLII
K_M (μM)	20.2 ± 0.9	14.3 ± 0.1	17.7 ± 0.2
V_{max} (IU × 10^{-3})	10 ± 0.8	8.2 ± 0.2	8.5 ± 0.1

in turn was calibrated against internal DSS. One-dimensional experiments were performed using the superWEFT pulse sequence (180°−τ−90°) *(39)*. Different delays (τ) were used in the superWEFT pulse sequence to optimize the detection of the fastest relaxing signals. The chemical shift values reported in the text were taken at 298 K. Nonselective T_1 values were determined by means of an inversion−recovery experiment. The T_1 values were calculated from the initial slope of the semilogarithmic plots of the fractional deviation of the *z*-magnetization from the equilibrium vs the intermediate delay τ *(40)*. The NMR data were processed on a PC-compatible computer using WinNMR software (Bruker).

RESULTS

Protein Expression and Purification. The strategy followed to construct the pETBLII expression vector for the production of *B. cereus* βLII in *E. coli* is summarized in Figure 2A. A *Bam*HI site was introduced in the −1 position by amplifying the cDNA of βLII (see Figure 2A and Experimental Procedures). The cDNA was ligated in phase with the 3′-end of the cDNA sequence for GST in a pETGEXCT plasmid *(32)*. The new plasmid (pETBLII) contains a sequence coding for a recognition site for thrombin cleavage between the GST and βLII sequences (Figure 2A).

The fusion protein GST−βLII accumulated to high yields in the host bacteria. The cell lysate was loaded onto a glutathione−agarose affinity column, which was thoroughly washed. Upon elution with glutathione, a single fraction could be recovered that was identified as a single band corresponding to a protein of molecular mass 51 kDa by SDS−PAGE (Figure 2B). This molecular mass agrees with that expected for the fusion protein from GST (26 kDa) and βLII (25 kDa). This protein was also recognized with antibodies specifically raised against both GST and βLII, and it exhibited GST and β-lactamase activity tested against nitrocefin, penicillin G and cephalosporin. This activity is inhibited when EDTA is added to the reaction mixture.

The purified fusion protein was treated with thrombin. The reported conditions for protease incubation correspond to the optimal situation in which disappearance of the band corresponding to the fusion protein is observed, and no truncated products are obtained. The proteolysis mixture, when analyzed by SDS−PAGE, showed only one broad band of ∼26 kDa (Figure 2B). Isoelectrofocusing analysis demonstrated that this band corresponded to two proteins with p*I* values of 6.1 and 8.3, which correspond to the reported values for GST and βLII, respectively (not shown) *(33)*. This protein mixture was purified by ion-exchange chromatography on Sephadex CM-50. Two major fractions were characterized. The first one was identified as GST, whereas the second one was characterized as βLII according to activity profile, SDS−PAGE migration pattern, and inmunoblotting analysis.

Recombinant βLII exhibited kinetic properties comparable with those of the native protein from *B. cereus* (see Table 1). Amino acid sequencing of the N-terminal region indicated the sequence GSQKVEKTVIKNE, which corresponds to the first 10 amino acids of mature *B. cereus* βLII *(17)* preceded by a Gly, as results from the introduction of the specific cleavage site for thrombin (Figure 2A), thus confirming the success of the chosen strategy.

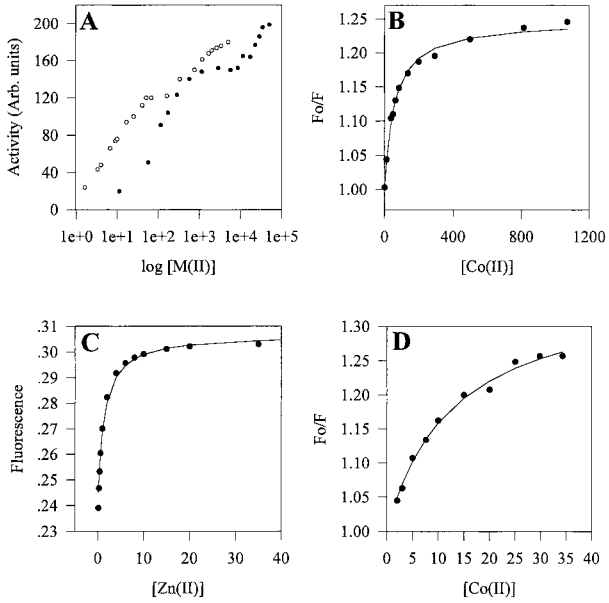

FIGURE 3: Zn(II) and Co(II) binding to apo-βLII. (A) Semilogarithmic plot showing the recovery of the β-lactamase activity upon titration of apo-βLII with Zn(II) (○) and Co(II) (●). The metal ion concentration is in micromolar units. (B) Fluorescence quenching upon addition of Co(II) to 0.5 μM apo-βLII until complete uptake of the first metal equivalent. (C) Fluorescence enhancement by addition of Zn(II) to 0.3 μM mono-Co(II)-βLII. (D) Fluorescence quenching upon addition of Co(II) to 0.5 μM mono-Zn-βLII until complete uptake of the second metal equivalent. F_0 represents the initial fluorescence, and F is the read value for each point. The continuous lines represent least-squares fits. The corresponding calculated K_D values are given in Table 2. These titrations were performed at pH 6.0 and 25 °C in 20 mM sodium succinate/1 M NaCl.

Metal Binding Studies. The apoenzyme is able to recover the activity of βLII by reincorporating Zn(II) *(7)*. The stepwise addition of zinc chloride allowed us to evaluate the dependence of the β-lactamase activity on the presence of increasing amounts of this metal ion. The data summarized in Figure 3A clearly show two inflection points. This situation is also met when Co(II) is added to apo-βLII (Figure 3A). Fitting of these curves to a hyperbolic equation yielded affinity constants of the apoenzyme for the two metal ions at both pH 6.0 and 7.0 (Table 2). These data somehow differ from others already reported *(7, 10, 41)*. According to those figures, it has been suggested that the observation of only one Zn(II) ion in the X-ray structure of βLII would be due to the looser binding to the second site *(13, 14)*. We decided to use other methods that provide structural information on the metal uptake.

Trp 59 and 189 are located at ca. 10 Å from Zn_1 in βLII *(12)*. If the fluorescence of these residues is affected by metal binding to the apoenzyme, this technique could provide a simple means to measure metal affinities independently of the activity of the metal sites *(42)*. The fluorescence emission spectrum of apo-βLII upon excitation at 286 nm yielded a maximum at 335 nm. Addition of saturating

Table 2: Thermodynamic Dissociation Constants of βLII for Zn(II) and Co(II) As Determined from Fluorescence and Activity Measurements

metal	pH	method[a]	K_D values
Zn(II)	6.0	A	$K_D^1 = 6 \pm 1\ \mu$M
			$K_D^2 = 760 \pm 100\ \mu$M
Zn(II)		B	$K_D^1 = 0.8 \pm 0.1\ \mu$M
			$K_D^2 = 2.5 \pm 0.2$ mM[b]
Zn(II)	7.0	A	$K_D^1 = 90 \pm 10\ \mu$M
			$K_D^2 = 1 \pm 0.1$ mM
Zn(II)		B	$K_D^1 = 120 \pm 10\ \mu$M
			$K_D^2 < 2$ mM
Co(II)	6.0	A	$K_D^1 = 95 \pm 10\ \mu$M
			$K_D^2 = 14 \pm 3$ mM
Co(II)		B	$K_D^1 = 110 \pm 10\ \mu$M
			$K_D^2 = 11.0 \pm 1$ mM[c]
Co(II)	7.0	A	$K_D^1 = 71 \pm 15\ \mu$M
			$K_D^2 < 14$ mM
Co(II)		B	$K_D^1 = 95 \pm 5\ \mu$M
			$K_D^2 = 3.7 \pm 0.5$ mM

[a] Method A, activity; method B, fluorescence (see text for details). [b] Measured by addition of Co(II) to the mono-Zn-βLII enzyme. [c] Measured by addition of Co(II) to the di-Zn(II)-substituted enzyme.

amounts of Zn(II) to the apoenzyme quenched only 13% of the fluorescence, as expected due to the diamagnetic nature of Zn(II). Efficient fluorescence quenching due to Zn(II) binding has only been observed in cases where severe conformational changes take place in the environment of the fluorophore upon metal binding *(42, 43)*.

Co(II) did efficiently quench the fluorescence of the apoenzyme to *ca.* one-third of its value. In this way, Co(II) binding can be monitored by this method. Addition of excess Zn(II) to Co(II)-βLII restores the fluorescence to *ca.* 80−90% of the value observed for holo-βLII, indicating a common interaction site. Hence, we could follow Zn(II) binding through the fluorescence recovery upon displacing Co(II) by back-titration. The metal uptake was shown to be reversible after Co(II) and Zn(II) binding by recovery of most of the fluorescence of apo-βLII upon treatment with EDTA. Representative binding curves are reported in Figure 3B−D, and the calculated dissociation constants are summarized in Table 2.

The titrations of apo-βLII against Co(II) at pH 6.0 and 7.0 show two clear inflection points, in agreement with those followed by activity measurements. A neat plateau is observed upon uptake of the first Co(II) equivalent (Figure 3B), thus allowing us to measure the affinity of the first site toward Zn(II) by metal displacement on the mono-Co(II)-βLII (Figure 3C).

To selectively titrate the low-affinity site, we took advantage of the distinct K_D values for the different metal ions (Table 2). Dialysis of βLII against a buffer solution at pH 6.0 containing 50 μM Zn(II) ensures an almost complete Zn(II) uptake at the first metal site, with the second one being empty. Addition of Co(II) to the mono-Zn βLII enzyme yields the hybrid ZnCo-βLII species, without displacing the Zn(II) ion from the first site (Figure 3D). The calculated K_D value agrees with the one retrieved from the second inflection in the direct titration of apo-βLII. The affinity of the second binding site toward Zn(II) was calculated by titrating ZnβLII in excess Zn(II) (10 mM) vs Co(II) (not shown). Again, the calculated values parallel those determined through activity measurements. This methodology

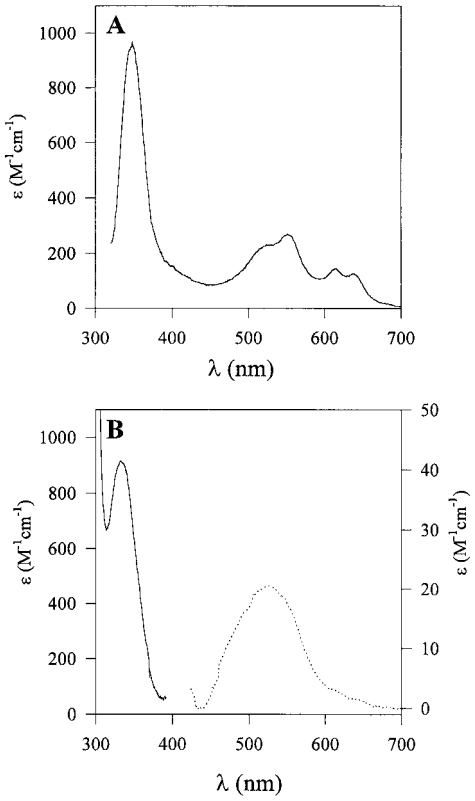

FIGURE 4: Electronic spectra of (A) CoCo-βLII and (B) ZnCo-βLII. In panel A, the enzyme concentration was 0.38 mM, and the total Co(II) concentration was 9 mM. The spectrum of the apoenzyme was subtracted. In panel B, the ZnCo-βLII species was obtained by addition of Co(II) to the mono-Zn-βLII adduct. The solid trace (at the left) corresponds to a sample of 0.15 mM enzyme reconstituted with 20 mM Co(II). The spectrum of mono-Zn-βLII was subtracted in the 300−400 nm region. The dotted trace (at the right) is the spectrum of 8 mM enzyme with 9 mM added Co(II). The spectrum of a reference Co(II) solution was subtracted, and the resulting spectrum was baseline-corrected for light scattering due to the high protein concentration. All the spectra were recorded at pH 6.0 in 20 mM sodium succinate/1 M NaCl.

proves to be useful for measuring metal binding affinities in βLII under conditions in which metal uptake cannot be monitored through hydrolytic activity.

Electronic Spectroscopy of Co(II)-βLII. The electronic spectrum of recombinant βLII upon addition of excess Co(II) is reported in Figure 4A, and it is identical to the reported spectrum of Co(II)-βLII from *B. cereus (7, 10, 11)*. An intense absorption is observed at 348 nm, being characteristic of a Cys → Co(II) LMCT band. Less intense features are observed in the visible region at 530, 551, 614, and 637 nm, attributed to ligand field transitions *(7, 10, 11)*. However, these spectral features have not been assigned to each one of the metal *loci*. We then recorded electronic spectra obtained by titrating apo-βLII with Co(II) (Figure 5A). As can be seen, different absorption spectra can be distinguished during the course of the titration. The Cys → Co(II) LMCT band is formed early in the titration (i.e., at low metal/protein ratios), simultaneously with a broad feature centered at 600 nm. The latter band disappears upon further addition of Co(II), giving rise to the four ligand field bands observed in the final adduct. The advent of these signals is concomitant with a substantial increase of the intensity of the 348 nm absorption (Figure 5). These spectral changes strongly suggest formation of a metal-protein adduct at low Co(II)

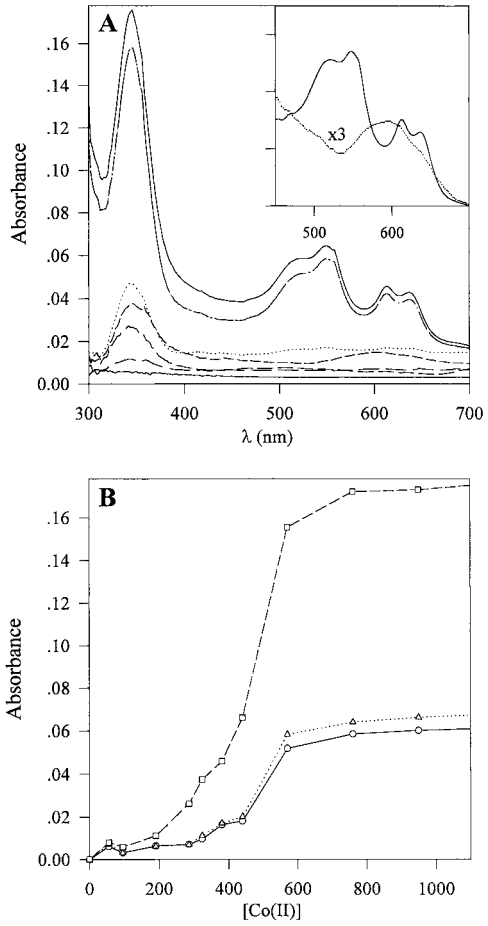

FIGURE 5: Evolution of the electronic spectra of apo-βLII in the presence of increasing Co(II) concentrations at pH 6.0. Panel A shows the recorded spectra corrected for dilution. The initial concentration of apo-βLII is 0.3 mM. The spectra correspond to added [Co(II)] of 0, 190, 285, 320, 380, 570, and 4000 μM. The inset shows the vertical expansion of the visible region of two points in the titration upon addition of 320 μM Co(II) (dotted line) and 570 μM Co(II) (solid line). Panel B shows the plot of the intensity of the different the electronic transitions vs [Co(II)] in micromolar units. The lines display the behavior of the 348 nm (□), 530 nm (○) and 551 nm (△) bands.

concentrations that later rearranges to form the final end-point complex at higher Co(II) concentrations.

The precedent titration, however, did not allow us to clearly differentiate the individual spectra of the first and second metal binding sites. We therefore obtained the mono-Zn-βLII enzyme (as already described), which was titrated with Co(II), in an attempt to obtain the ZnCo-βLII adduct. Saturation of the second metal site with Co(II) yielded a spectrum with the LMCT band but without the four-band ligand field pattern present in the CoCo-βLII enzyme (Figure 4B). This spectrum allows us to ascribe the LMCT band to the low-affinity binding site. The detection of ligand field bands was obscured by the absorption of excess free Co(II) at 512 nm. Titrations performed on concentrated samples (8 mM) of mono-Zn-βLII revealed a weak, broad absorption centered at 520 nm, which we assign as arising from d−d transitions of this species (Figure 4B). A calculation of the amount of bound Co(II) according to the determined K_D values (Table 2) allowed us to estimate a value of $\epsilon \approx 20$ M^{-1} cm^{-1} for this composite band.

NMR Study of Co(II)-βLII. Paramagnetic NMR is a useful spectroscopic tool for characterizing metal sites in proteins,

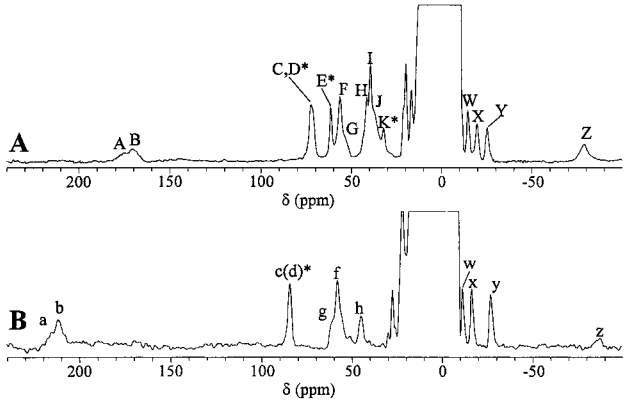

FIGURE 6: ^{1}H NMR spectra of (A) CoCo-βLII, and (B) ZnCo-βLII. Signals marked with an asterisk are absent when the spectra are recorded in D_2O. The spectra were recorded in H_2O at 200 MHz and 298 K in 20 mM sodium succinate/1 M NaCl at pH 6.0.

since it is able to reveal the identity of unknown metal ligands *(44−46)*. The ^{1}H NMR spectra of the CoCo-βLII and ZnCo-βLII adducts were recorded in conditions suitable for the detection of paramagnetically shifted signals. A number of hyperfine shifted signals spanning from 240 to −90 ppm were observed in both metal derivatives (Figure 6), attributable to protons belonging either to Co(II) ligands or to residues in the near environment of the paramagnetic ion *(44−46)*. The observed shifts and relaxivity features are typical of paramagnetic Co(II) metalloproteins *(44−46)*. In particular, signals A−K are well shifted and display short T_1 values, indicating that they correspond to protons belonging to Co(II) ligands, experiencing nonnull contact shifts. All the hyperfine resonances broaden at higher magnetic fields, thus indicating the existence of a Curie relaxation mechanism *(47)*. For this reason, it was more appropriate to study the hyperfine shifted resonances at 200 MHz.

A comparative analysis of the spectra shows that a number of resonances are absent in the ZnCo-βLII spectrum, as expected. They can be readily assigned as corresponding to the metal ligands of the high-affinity site. The remaining resonances exhibit slightly different shifts in the two species (except signals A and B) and may be tentatively correlated according to their chemical shifts (Figure 6). Resonances C, D, E, and K are absent in spectra of CoCo-βLII recorded in D_2O, thus indicating that they correspond to solvent-exchangeable protons. This fact and their shifts allow us to assign them to imidazolic NH His protons. Only one of these resonances is present in the spectrum of ZnCo-βLII (C or D), thus confirming the presence of one His ligand in the low-affinity site. These observations concur with the His ligands observed in the crystal structure of the *B. fragilis* enzyme (Figure 1B) *(13−15)*.

The large chemical shifts, very short T_1 values, and broad line widths of signals A and B are characteristic of the β-CH_2s of a Co(II)-bound Cys *(22, 47−51)*, now clearly identified as a ligand of the low-affinity site, in agreement with the electronic spectra. According to their spectral features, resonances F, G, and H correspond to a metal ligand of the low-affinity site. Finally, the remaining signals display smaller chemical shifts and longer T_1 values and are attributed to nonbound residues located in the neighborhood of the metal site. The thorough assignment of these resonances was attempted through NOE experiments, which

proved unsuccessful in all cases due to the low signal-to-noise ratio of the 1D spectra and to the signal broadening attributable to protein aggregation *(16)*.

DISCUSSION

The present results clearly demonstrate that apo-βLII is able to bind competitively two metal ion equivalents of both Zn(II) and Co(II). Recombinant βLII is identical to the *B. cereus* enzyme according to all the reported data, thus allowing us to extrapolate the structural information here discussed. Zn(II) binds more tightly than Co(II) to both sites. However, Co(II) is able to compete more efficiently in binding to the second site. Co(II) uptake monitored by electronic spectroscopy revealed the formation of a tetrahedral adduct, which later rearranges to yield the resting state conformation (Figure 5A). This spectrum resembles the one of an enzyme−substrate intermediate detected in low-temperature kinetic studies of Co(II)-βLII *(11)*. This observation suggests a certain degree of flexibility in the metal site, which may be of functional relevance.

The spectrum of Co(II)-βLII in the visible region is remarkably similar to that of Co(II)-carbonic anhydrase at alkaline pH, as already noted *(11)*. The intensity of the ligand field bands in Co(II)-substituted enzymes can be analyzed as a fingerprint of the metal-site geometry, and similar spectra are expected to reflect similar coordination environments *(18, 19)*. The only metal site found in the crystal structure of βLII and the Zn_1 site in the *B. fragilis* enzyme are almost superimposable with the Zn(II) site in carbonic anhydrase *(52)*, i.e., a His_3ZnOH polyhedron. These intense d−d bands can then be attributed to a Co(II) ion coordinated to three His and a water/hydroxyl molecule with a tetrahedral geometry, which corresponds to the high-affinity metal site in βLII. This assertion is validated by the finding that incorporation of Co(II) when the first site is occupied by Zn(II) does not give rise to these bands (cf. the spectrum of the ZnCo-βLII adduct, Figure 4B). The visible spectrum of alkaline Co(II)-carbonic anhydrase has been interpreted as arising from a distorted tetrahedral environment *(53)*. This distortion is dictated by a strong hydrogen bond between the metal-bound solvent molecule and a Thr residue *(52)*, which confers on the active hydroxyl the right orientation for the nucleophilic attack *(54)*. This distortion is also present in Co(II)-βLII, as apparent from the spectral data, and may also obey functional requirements. The electronic spectrum of the CoCo-β-lactamase from *B. fragilis* displays less-resolved and weaker d−d transitions in the visible region without evidence of a tetrahedral Co(II) site *(16)*.

The low-affinity site is characterized by a Cys and a His ligand, as clearly identified from the spectral data. A third protein ligand has been identified through NMR (signals G, F, and H), and taking into account the resemblance of this site to the one of the *B. fragilis* enzyme, it may well be an Asp residue. These signals display shifts in the range corresponding to the β-CH_2 and α-CH protons of a Co(II)-bound Asp *(50, 55−57)*. The observation of weak ligand field bands in the visible spectrum corresponding to the low-affinity binding site (Figure 4B) may be indicative of an octahedral coordination for Co(II) *(18, 19)*. Taking into account that Zn_2 is pentacoordinated in the *B. fragilis* enzyme and that we have not found evidence of additional protein ligands, this may be explained by assuming that two nonbridging water molecules are coordinating Co_2 in βLII. An additional solvent ligand has been observed in Co(II) thermolysin with respect to the Zn(II) enzyme *(25)*. On the other hand, theoretical calculations have claimed that in some cases pentacoordinated Co(II) may give rise to low-intensity ligand field bands *(53)*, resembling the spectra of octahedral species, so that this possibility cannot be discarded.

By and large, this overall picture resembles the binuclear metal site of the *B. fragilis* β-lactamase (Figure 1B) *(13−15)*. Thus, we feel confident to extrapolate this conclusion to the diZn(II)-βLII. This structural similarity explains why Co(II)-βLII is able to mimic the native enzyme activity. It is surprising to note that the X-ray structure of the Zn(II) enzyme from *B. fragilis* agrees more with the structure arising from the spectral features of CoCo-βLII than with the ones coming from its own Co(II) derivative *(16)*.

CONCLUDING REMARKS

We have taken advantage of the distinct affinities of the two metal sites in βLII and succeeded in obtaining individual structural pictures of both of them. The binding of the first metal equivalent yields an active enzyme, which is even more efficient upon binding of the second metal ion. The metal ion could display a 2-fold functional role: (1) to "lock" the bridging hydroxyl into an orientation appropriate for the nucleophilic attack to the β-lactam ring (like Thr199 in carbonic anhydrase) and (2) to enhance the nucleophilicity of this hydroxyl group. Such an enhancement has recently been observed in a binuclear Co(III) model complex *(58)*. Under this hypothesis, this bridging hydroxide is expected to be the active nucleophile, as proposed for the dizinc enzyme leucine aminopeptidase *(59, 60)*. The solvent molecule bound to Zn_2 instead may act as a proton donor. This mechanism could stand for both the *B. cereus* and the *B. fragilis* enzymes, and the differences revealed by the crystal structures may only reflect different metal affinities, as already noted *(13)*. In this way, the presence of a second metal binding site in βLII may not be fortuitous.

The present results allow us to place βLII in particular and metallo-β-lactamases in general in the family of dizinc hydrolases *(61)*. The β-lactamase from *Aeromonas hydrophila* (which exhibits only carbapenemase activity) is also able to bind two metal ions but displays a distinct behavior, since the second equivalent acts as an inhibitor *(62)*. Curiously, the three enzymes display a high degree of homology in their putative active sites. Further studies on this family of enzymes could reveal particulars on the fine-tuning of the metallo-β-lactamase activity via point mutations on the active-site environment.

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