Purification, Characterization, and Kinetic Studies of a Soluble Bacteroides fragilis Metallo-β-lactamase That Provides Multiple Antibiotic Resistance*

(Received for publication, March 12, 1998, and in revised form, June 5, 1998)

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Resistance to multiple β-lactam antibiotics traced to the expression of Zn(II) requiring metallo-β-lactamases has emerged in clinical isolates of several bacterial strains including Bacteroides fragilis, a pathogen commonly found in suppurative/surgical infections. A soluble B. fragilis metallo-β-lactamase has been purified to homogeneity from the cell growth medium after expression as a secretory protein in Escherichia coli. The enzyme requires two tightly bound Zn(II) ions for full activity, and the Zn(II) ions can be removed by EDTA from the enzyme. The apoenzyme is reactivated by stoichiometric amounts of Zn(II) and Co(II) ions. The Co(II)-substituted enzyme exhibits a UV-visible spectrum characterized by strong Co(II) d-d transitions at 510, 548, 615, and 635 nm and an EPR spectrum with g values of 5.52, 4.25, and 2.01: features that serve as useful spectroscopic handles for the mechanistic studies of the enzyme. Although steady-state and transient-state kinetic studies of the soluble Zn(II) enzyme with nitrocefin as substrate found no ionizable groups with pKₐ values between 5.25 and 10.0 involved in catalysis, a kinetically significant proton transfer step in turnover was implicated by studies in deuterium oxide. These studies also detected the accumulation of an enzyme-bound intermediate and provide the basis for a minimal kinetic scheme describing metallo-β-lactamase-catalyzed nitrocefin hydrolysis.

The widespread use of antibiotics has put tremendous selective pressure on bacteria to devise mechanisms to escape the lethal action of the drugs. The most common and efficient result is a loss of enzymatic activity (23). It seems that metallo-β-lactamases from different sources have differing Zn(II) requirements, which may account for the wide range of catalytic efficiencies of different metallo-β-lactamases (24).

B. fragilis is one of the most important pathogens in polymicrobial infections in humans (25–27). Recently, a clinical isolate of B. fragilis expressing a metallo-β-lactamase proved resistant to multiple β-lactam antibiotics (28), underscoring the need for clinically useful inhibitors of the enzyme. One impor-
tart step to aid in this task is to elucidate the catalytic mechanism of this enzyme. However, the B. fragilis metallo-β-lactamase currently used in biochemical and structural studies (19, 22, 29) was produced as inclusion bodies in E. coli. The active enzyme is obtained by renaturing the 8 M urea-solubilized inclusion bodies in metal ion-containing buffer (Zn(II) or Co(II)). The preparation is subject to aggregation at high protein concentration and is not reliably reproducible.1 The purified Co(II) enzyme failed to show the desired spectroscopic features of a Co(II) enzyme (22). Here, we report the subcloning, overexpression, and purification of a soluble B. fragilis metallo-β-lactamase. Using the soluble enzyme, we were able to characterize the effects of Zn(II) on activity, to prepare a Co(II)-containing enzyme whose spectroscopic features further define the metal ion binding sites, and to establish the minimum kinetic sequence for the enzyme-catalyzed nitrocefin hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials—** E. coli strain DH5α was obtained from Life Technologies, Inc. E. coli strain BL21(DE3) and plasmid pET-27b(+) were from Novagen. Plasmid pET-CcrANDE02 (having a BamHI and NcoI site), was de-

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Characterization of Soluble Metallo-\(\beta\)-lactamase

**RESULTS**

**Overexpression and Purification—*B. fragilis* metallo-\(\beta\)-lactamase** is a secretory protein with its leader sequence comprising amino acid residues 1–17 (13, 29). Our plan was to subclone the leaderless *B. fragilis* metallo-\(\beta\)-lactamase gene (encoding amino acids 18–249) into plasmid pET-27b(+) so that the truncated gene is fused with a *pelB* leader sequence under control of a bacteriophage T7 promoter. As a result, the overexpressed metallo-\(\beta\)-lactamase has the potential to be secreted into the periplasm in a soluble form. The construction of the expression plasmid, pMSZ02, is quite straightforward (Scheme 1). To overexpress the metallo-\(\beta\)-lactamase, we first grew the pMSZ02-transformed *E. coli* BL21(DE3) strain in rich medium to the midlog phase (OD 600nm = 0.6) at 37 °C. The cell culture was then cooled to 25 °C and induced with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. The induction was performed at 25 °C to avoid cell lysis (34). However, very little soluble protein was found in the periplasmic space after induction; most appeared in the growth medium. The production of the soluble protein reaches a maximum after 18–20 h of induction and accounts for more than 90% of the total protein in the medium (Fig. 1). The \(\beta\)-lactamase activity in the cell growth medium was determined during the induction and found proportional to the level of the soluble enzyme produced (data not shown). The fact that the \(\beta\)-lactamase activity in the medium was inhibited by EDTA in a time-dependent fashion (data not shown) demonstrates that the soluble enzyme is indeed a metallo-\(\beta\)-lactamase. During the expression, large amounts of insoluble metallo-\(\beta\)-lactamase also accumulated in the cytoplasm.

The secretion of the enzyme into cell growth medium makes its purification relatively easy (Fig. 1). Minitan concentrators proved very efficient in concentrating the medium; four 10,000 NMWL membranes concentrated 4 liters of medium to 50 ml in about 6 h. After further concentration with an Amicon YM-10 membrane, the protein solution was dialyzed to adjust pH and to lower the salt concentration. A Q-Sepharose column was used to remove most of the positively charged components in the medium and the metallo-\(\beta\)-lactamase-containing fractions eluted using a linear salt gradient between 0.15 and 0.2 M NaCl. Highly purified metallo-\(\beta\)-lactamase was obtained after a final Sephadex G-75 gel filtration column (Fig. 1). We have consistently isolated 80–100 mg of purified enzyme from 4 liters of cell culture.

**Properties of the Soluble Metallo-\(\beta\)-lactamase**—As expected, the purified soluble metallo-\(\beta\)-lactamase has a molecular mass of ~26 kDa (Fig. 1). Atomic absorption measurements revealed that the soluble metallo-\(\beta\)-lactamase contained two Zn(II) ions per protein molecule (Table I), and no other metal ions were found in significant amounts (data not shown). With nitrocefin as the substrate, the soluble metallo-\(\beta\)-lactamase exhibited a \(K_m\) value similar to that of the renatured enzyme that had been purified from urea-solubilized inclusion bodies (Table I). However, the specific activity of the soluble enzyme is much higher than that of the renatured enzyme; the \(k_{cat}\) value of the soluble enzyme is higher than 200 s\(^{-1}\), whereas our best \(k_{cat}\) value for the renatured enzyme is only 160 s\(^{-1}\) at pH 7.0 (Table I).

Like the renatured enzyme (22), the soluble metallo-\(\beta\)-lactamase can be inactivated by Zn(II) chelating agents such as EDTA and 1,10-phenanthroline in a time-dependent manner. Attempted preparation of the apoenzyme by dialyzing the purified Zn(II)-\(\beta\)-lactamase against 2 mM 1,10-phenanthroline-containing buffer at pH 7.6 resulted in the precipitation of the protein. However, dialysis of the Zn(II)-\(\beta\)-lactamase against 10 mM EDTA-containing buffer followed by extensive dialysis against metal-free buffer to remove EDTA provided the apoenzyme with almost no activity (Fig. 2). When increasing Zn(II) concentrations were added to the apoenzyme, the initial hydrolysis rate of nitrocefin markedly increased. About 80% of the enzymatic activity was restored after 1 equivalent of Zn(II) was introduced and full enzymatic activity recovered after 2 equivalents of Zn(II) were added to the apoenzyme (Fig. 2). This result reveals an important difference between the soluble enzyme and the renatured enzyme; the renatured enzyme cannot be reactivated by any metal ions after being treated with chelating agents (22).

**Reconstitution of the Aopoenzyme with Other Metal Ions**—Co(II) can reactivate the apoenzyme with maximal activity reached at two equivalents (Fig. 2). However, the Co(II)-substituted enzyme possesses only half of the activity of the Zn(II) enzyme (Fig. 2 and Table I). Slight activity, approximately 4% and 8%, was restored with Cd(II) and Mn(II), respectively, but no activity was recovered by adding Ni(II) and Cu(II) to the apoenzyme. Metal analysis confirmed the stoichiometric incorporation of Cd(II) and Mn(II) into the apoenzyme, whereas no bound metal ions were found in the Ni(II)- and Cu(II)-treated protein.
Spectrophotometric titration of the apoenzyme with increments of Co(II), accompanied by scanning of the absorbance spectrum, confirmed that there are two Co(II) binding sites in the enzyme (Fig. 3). The addition of one equivalent of Co(II) resulted in most of the spectral changes, whereas the addition of the second equivalent of Co(II) completed the spectral changes. The UV-visible difference spectrum of the soluble Co(II) enzyme (Fig. 4, spectrum of soluble Co(II) enzyme minus the spectrum of the apoenzyme) has five features: an intense S-to-Co(II) ligand to metal charge transfer transition (LMCT) band at 340 nm ($\epsilon = 820 \text{ M}^{-1}\text{cm}^{-1}$) and four Co(II) d-d transitions at 510, 548, 615, and 635 nm ($\epsilon = 150, 230, 210, \text{ and } 190 \text{ M}^{-1}\text{cm}^{-1}$, respectively) (35, 36). This is very different from the UV-visible spectrum of the apoenzyme, which failed to show the characteristic features of the Co(II) d-d transitions (22).

The low temperature EPR spectrum of the Co(II)-substituted $\beta$-lactamase is similar to the one obtained for the renatured enzyme (22). It is characterized by signals with apparent $g$ values of 5.22, 4.25, and 2.01 (Fig. 5), typical of $S = 3/2$ high spin, mononuclear Co(II) species. The EPR signal is integrated to 1.7 mol of Co(II)/mol of enzyme using a CoCl$_2$ sample as standard. This indicates that there are two Co(II)-binding sites in the protein and the two Co(II) ions are both high spin and do not interact with each other to form a magnetically coupled dinuclear site. The fact that the signal was temperature-dependent and disappeared at temperatures higher than 50 K (data not shown) supports the above conclusion.

**Kinetic Studies of the Soluble Zn(II)-$\beta$-Lactamase**—In order to obtain accurate kinetic parameters, 1× MTEN buffer, a three-buffer system known to have constant ionic strength through a wide pH range (37), was chosen for the kinetic studies. The $K_m$ and $k_{cat}$ values of the Zn(II)-$\beta$-lactamase-catalyzed nitrocefin hydrolysis were determined at different pH values. The pH dependence of the logarithm of $k_{cat}$ and $k_{cat}/K_m$ of the soluble Zn(II)-$\beta$-lactamase is presented graphically in Fig. 6. Both curves are rather flat between pH 5.25 and 10.0. As the enzyme loses activity rapidly at low pH and the background hydrolysis of nitrocefin is too fast at high pH, we were unable to perform studies at pH values under 5.25 and above 10.0. It therefore appears that there are no ionizable groups with $pK_a$ values between 5.25 and 10.0 involved in the catalysis.

The pH dependence of $k_{cat}$ and $k_{cat}/K_m$ for the soluble Zn(II)-$\beta$-lactamase-catalyzed nitrocefin hydrolysis was also studied in D$_2$O. Both $k_{cat}$ and $K_m$ values decreased when the reactions were carried out in D$_2$O. The kinetic solvent isotope effect on $k_{cat}$ ranges from 2.35 to 2.79 and on $k_{cat}/K_m$ is $\approx 1.6$ from pH/pD 6.0 to pH/pD 10.0 (Table I). These results are in accord with a proton transfer in a kinetically significant step of the reaction.
The catalytic activity of the soluble Zn(II)-β-lactamase was also examined in detail by a stopped-flow method to determine whether any enzyme-bound intermediate could be detected. Fig. 7 shows the consumption of nitrocefin and the formation of the product during a single turnover experiment using 25 mM Zn(II) enzyme and 5 mM substrate at pH 5.5. The data fit well to a single exponential with no sign of a large burst or lag phase with rate constants of 900 s⁻¹ for the consumption of the substrate and 150 s⁻¹ for the formation of the product. The large difference between the two rates suggests that the consumption of the substrate and the formation of the product proceed through an intermediate that accumulates during the turnover. A plot of the deficit indicated the transient formation of an intermediate of up to 3.4 mM. One model for the kinetic scheme to accommodate the above observation is shown in Scheme 2. This minimal model involves four steps: substrate binding, formation of the intermediate (EI), breakdown of the intermediate to form the product, and product release. In order to show that the kinetic mechanism is valid, the three sets of data points representing [S] = [E], [E], and [EP] + [P] in Fig. 7 were simulated using the program KINSIM with the rate constants summarized in Table III. All three data sets can be well simulated by the same parameters, which in turn provide the calculated kcat and Km values close to those obtained from the steady-state kinetic studies (Table IV).

The consumption of nitrocefin and the formation of the product during a single turnover experiment using 25 µM Zn(II) enzyme and 5 µM substrate were also studied at pH 7.0 and pD 7.0 (data not shown). The formation of an intermediate during the reaction was also evident under these conditions. Using the same kinetic mechanism (Scheme 2), the data were well simulated by the parameters listed in Table III. Therefore, Scheme 2 most likely represents the minimum kinetic sequence of the Zn(II)-β-lactamase-catalyzed nitrocefin hydrolysis where breakdown of the intermediate is the rate-determining step.
DISCUSSION

By subcloning the gene encoding amino acids 18–249 of the *B. fragilis* metallo-β-lactamase in frame with the T7 *pelB* sequence in the pET-27b(+) vector, we were able to overexpress the enzyme very efficiently in *E. coli* BL21(DE3). A portion of the overexpressed enzyme was subsequently processed and secreted into the cell growth medium to yield an active, soluble metallo-β-lactamase lacking the putative signal sequence. The yield of the soluble enzyme is thus modest, but it accounts for more than 90% of the total protein in the medium (Fig. 1), simplifying purification. The purified enzyme binds 2 mol of Zn(II)/mol of protein and exhibits higher catalytic activity than the renatured enzyme, indicating that the soluble enzyme is properly folded. This is further supported by the fact that the bound Zn(II) ions can be removed to form apoenzyme and the apoenzyme reconstituted with Zn(II) to recover full enzymatic activity. The apoenzyme derived from the renatured enzyme can not be reconstituted (22). Recently, Toney and co-workers reported another system for expressing the soluble *B. fragilis* metallo-β-lactamase in *E. coli*, but no information on whether the apoenzyme could be generated and the native enzyme then reconstituted was provided (39).

One powerful means to study Zn(II) enzymes is to substitute the Zn(II) ions with Co(II). Co(II)-substituted Zn(II) enzymes are usually active and exhibit rich spectroscopic features that are lacking in the native enzymes. However, the Co(II)-substituted enzyme made from the renatured *B. fragilis* metallo-β-lactamase failed to show any distinct features in the Co(II) d-d transition region in its UV-visible spectrum (22). The soluble enzyme provided Co(II)-substituted *B. fragilis* metallo-β-lactamase suitable for spectroscopic studies. The enzyme reconstituted from Co(II) binds 2 mol of Co(II)/mol of enzyme, retains roughly half of the activity of the Zn(II) enzyme, and has an activity-Co(II) dependence very similar to that of the Zn(II) enzyme (Table I and Fig. 2). The UV-visible spectrum of the Co(II)-substituted enzyme has five distinct features: an intense S-to-Co(II) LMCT band at 340 nm and four Co(II) d-d transition bands at 510, 548, 615, and 635 nm (Fig. 4). The 340-nm LMCT band is due to the Cys-168 ligation to Co(II), as demonstrated in the x-ray crystal structure (19). The pattern and intensities of the d-d transition bands are in agreement with the existence of two distinct Co(II) binding sites with distorted tetrahedral and trigonal bipyramidal coordination geometries, respectively, in agreement with the crystal structure of the Zn(II) enzyme (19). The fact that all the UV-visible signals appear simultaneously upon the addition of Co(II) (Fig. 3) indicates that both sites have similar binding affinities for the metal ions. Despite the difference between their UV-visible spectra, the Co(II)-substituted renatured enzyme and soluble enzyme exhibit similar low temperature EPR spectra. The EPR spectrum suggests that the two Co(II) ions in the Co(II) proteins are both high spin and not spin-coupled.

The UV-visible spectrum of nitrocefin has an intense absorbance at 390 nm, which is shifted to 485 nm upon hydrolysis (31). The absence of spectral overlap between nitrocefin and its hydrolysis product makes it an ideal substrate for mechanistic studies of β-lactamases. The *B. fragilis* Zn(II)-β-lactamase-catalyzed nitrocefin hydrolysis is rapid and irreversible. During nitrocefin hydrolysis, the reaction velocity can be determined from either the time-dependent decrease of the absorbance at 390 nm (substrate disappearance) or the time-dependent increase of the absorbance at 485 nm (product formation). As both Zn(II) ions in the dinuclear Zn(II) center of the *B. fragilis* metallo-β-lactamase are required for the maximum enzymatic activity, a possible mechanism for the breakdown of the β-lactam ring is that the carbonyl group activated by one Zn(II) is attacked by a nucleophile followed by breaking of the C-N bond. The fact that $k_{cat}$ and $k_{cat}/K_m$ have no pH dependence suggests that the pKₐ value of the nucleophile is lower than 5.25 or higher than 10.0. A possible candidate for the nucleophile is believed to be the water molecule shared by both Zn(II) ions (19). The pKₐ value of Zn(II)-bound water is usually between 6 and 10. However, the combination of the hydrogen bond between the bridging water and the carboxylate of Asp-90, the dimetal coordination, and the dipositive effective charge of the Zn(II) site with three histidine ligands may perturb the pKₐ value of the bridging water to below 5.25 (40, 41).

Stopped-flow measurements of single turnover that measured substrate disappearance and product formation were consistent with the accumulation of an enzyme-bound intermediate at pH 5.5, pH 7.0, and pH 7.0. From the simulated kinetic constants, the bindings of substrate and product are under diffusion control. The attack step to form EI designated as $k_2$ is at least seven-fold greater than its breakdown through step $k_3$. The step for formation of the intermediate is pH- and D₂O-insensitive, whereas the slight pH dependence of $k_{cat}$ and $K_m$ (Table IV) is due primarily to variations in the rate of the breakdown step. In addition, the observed solvent isotope effects (Table II) provide evidence for the involvement of a proton transfer event in the rate-determining step, i.e., the breakdown of the intermediate.

On the basis of the kinetic sequence and chemical precedents, one can envision that, upon binding, the carbonyl group of the β-lactam ring is activated for cleavage through polarization by one Zn(II) ion followed by attack of the hydroxide.

### Table III

| Constants | pH 5.5 | pH 7.0 | pD 7.0 |
|-----------|--------|--------|--------|
| $k_1$ (M⁻¹s⁻¹) | $1 \times 10^8$ | $1 \times 10^9$ | $1 \times 10^8$ |
| $k_2$ (s⁻¹) | 3000 | 5000 | 7000 |
| $k_3$ (s⁻¹) | 2400 | 2400 | 2400 |
| $k_4$ (s⁻¹) | 160 | 340 | 130 |
| $k_{-4}$ (M⁻¹s⁻¹) | $1 \times 10^{10}$ | $1 \times 10^8$ | $1 \times 10^8$ |

**Scheme 2.**

### Table IV

| Constants | pH 5.5 | pH 7.0 | pD 7.0 |
|-----------|--------|--------|--------|
| $k_{cat}$ (s⁻¹) | 147.7 | 132.3 | 297.8 |
| $K_M$ (μM) | 2.0 | 2.6 ± 0.4 | 7.1 |

$$K_M = \frac{k_3}{k_2 + k_3}; \quad K_m = \frac{k_{cat}}{k_2 + k_{cat}}.$$
derived from the bridging water to form an acyl-enzyme intermediate. Collapse of this species may proceed through displacement by water of the cleaved lactam and regeneration of the bridged dinuclear active site. This latter step appears to have an associated proton transfer. Alternatively, the enzyme nucleophile may be derived from one of the side chain residues acting as ligand to the Zn(II) ions. Additional experiments, especially spectroscopic characterization of the intermediate are needed to more fully elucidate the catalytic mechanism. 3

The active site of the B. fragilis metallo-beta-lactamase possesses the two conserved functional groups, i.e. a water molecule bridging the two Zn(II) ions and a carboxylate group hydrogen-bonded to this water, found in all the dizinc peptidases (41). This structural feature facilitates the deprotonation of the bridging water molecule, transforming it into a potent nucleophile at neutral pH, and acts to activate the substrate by polarizing its carbonyl group. The Zn(II) ions may also contribute to the electrophilic stabilization of the acyl intermediate and the flanking transition states. A deeper understanding of the catalytic mechanism of the B. fragilis metallo-beta-lactamase will not only provide vital information for the development of clinically useful inhibitors, but will also generate insights into the catalytic mechanism of other bimetallopeptidases.

Acknowledgments—We are grateful to Drs. Michael W. Crowder and Craig E. Cameron for all the helpful discussions and suggestions and to Fan Yang for collecting the EPR spectrum. We thank Dr. Beth Rasmussen at American Cyanimid Co. for the pT7CcrANDE02 clone, Dr. John Gleason at SmithKline Beecham for providing nitrocefin, and Dr. Henry Gong at the Pennsylvania State University Materials Characterization Laboratory for helping in the metal content measurements.

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3 Preliminary results revealed that the intermediate is associated with an absorbance maximum at ~665 nm, thus ruling against a tetrahedral species. Recently, this intermediate has also been detected in the S. maltophilia L1 metallo-beta-lactamase-catalyzed hydrolysis of nitrocefin by another group (M. W. Crowder, personal communication).