Direct Observation of an Enzyme-Bound Intermediate in the Catalytic Cycle of the Metallo-β-Lactamase from *Bacteroides fragilis*

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The emergence of simultaneous resistance to all available β-lactam antibiotics through synthesis of metallo-β-lactamases by some pathogenic bacterial strains has caused great concerns in the medical community.[1] Understanding the catalytic mechanism of metallo-β-lactamases is an important first step toward the development of new antibiotics. Our focus has been on the metallo-β-lactamase from *Bacteroides fragilis*,[2] a pathogen commonly found in suppurative/surgical infections.[3] Recent metal-binding, spectroscopic, and X-ray crystallographic studies have yielded clear pictures of the overall structure and the dinuclear Zn(II) active site of this enzyme.[4,5] With single-wavelength stopped-flow analysis using nitrocefin,[6] (6*R*)-3-[(2,4-dinitro)-styryl]-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid, as a substrate, we established the minimal kinetic mechanism of the *B. fragilis* metallo-β-lactamase (Scheme 1).[7] Here, we report a direct observation of the accumulation of an enzyme-bound intermediate during the *B. fragilis* metallo-β-lactamase-catalyzed nitrocefin hydrolysis by using a rapid scanning stopped-flow technique. The intermediate has an intense absorbance band at 665 nm and is most likely an acyl intermediate with a negatively charged nitrogen formed upon cleavage of the β-lactam.

In a typical experiment, soluble *B. fragilis* metallo-β-lactamase[7] was rapidly mixed with nitrocefin in 1X MTEN (50 mM Mes, 25 mM Tris, 25 mM ethanol amine, 100 mM NaCl), pH 7.0 buffer at 25 °C in an Applied Photophysics SX.18MV stopped-flow spectrometer. The absorbance changes during the reaction were monitored with an Applied Photophysics PD.1 photodiode array detector. Figure 1 shows a set of scans taken every 2.56 ms (starting at 1.28 ms after mixing) in the wavelength range of 310 to 725 nm during the reaction of 5 μM enzyme with 10 μM nitrocefin. The absorbance decrease at 390 nm represents the consumption of the substrate, whereas the absorbance increase at 490 nm corresponds to the formation of the product.[6] However, product formation does not simultaneously accompany the substrate consumption. Indeed, there is a new absorbance feature at 665 nm which increases rapidly then decreases at a slower rate (Figure 1). Single-wavelength stopped-flow analysis carried out at 390, 490, and 665 nm confirmed this observation (data not shown). This is direct evidence that an enzyme-bound intermediate accumulates during the reaction, in agreement with our previous conclusion.[7] Since the enzyme is spectrally transparent above 310 nm and no absorbance features other than those at 390, 490, and 665 nm were observed during the reaction, we assume that the Michaelis complexes, ES and EP, have the same visible absorbance spectra as S and P, respectively. Global analysis[8] of the absorbance changes at all wavelengths collected (310 to 725 nm) using the Applied Photophysics !ProK software yielded absorption spectra for the initial (S and ES), intermediate (EI), and final species (EP and P) (See Supporting Information) and the kinetic parameters (Table 1) for the four-step mechanism (Scheme 1). The absorbance spectrum of the intermediate has a very intense absorbance band at 665 nm with a molar coefficient of 30 000 M^{-1} cm^{-1}. Kinetic parameters obtained from global analysis are similar to those derived from simulation of the single-wavelength stopped-flow data using KINSIM (Table 1),[7] suggesting that the rate-determining step of the reaction is the breakdown of the intermediate.

The 390-nm visible absorbance band of nitrocefin is due to the conjugation of the 2,4-dinitrostyryl substituent in the 3-position with the double bond of the dihydrothiazine ring. The hydrolysis product of nitrocefin has the C−N bond of the β-lactam ring cleaved by the addition of a water molecule.[6,9] The red-shift of the absorbance band (to 490 nm) in the product is due to the rupture of the β-lactam ring. However, the marked absorbance

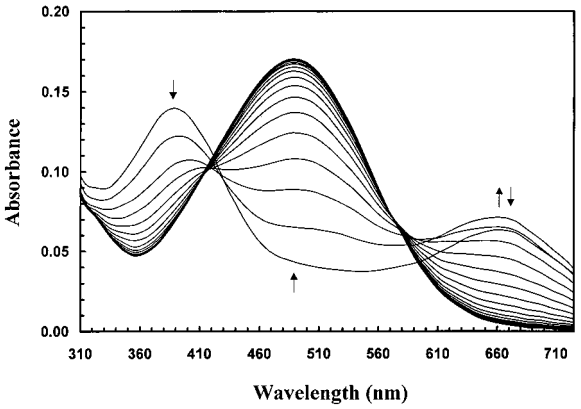

Figure 1. Spectral changes during the *B. fragilis* metallo-β-lactamase (5 μM)-catalyzed nitrocefin (10 μM) hydrolysis at pH 7.0 and 25 °C. The first spectrum was taken 1.28 ms after mixing and the rest collected every 2.56 ms thereafter. The reaction was complete in 50 ms, and thus, only the spectra from the first 20 scans were displayed. The absorbance changes at 390, 490, and 665 nm are indicated with arrows.

Scheme 1

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{EI} \xrightarrow{k_3} \mathrm{EP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E + P}$$

Table 1. Kinetic Constants of the *B. fragilis* Metallo-β-Lactamase-Catalyzed Nitrocefin Hydrolysis at pH 7.0 and 25 °C

constants	from KINSIM simulation[a]	from global analysis[b]
k_1 (M^{-1} s^{-1})	1×10^8	1×10^8
k_{-1} (s^{-1})	5000	3800−5000
k_2 (s^{-1})	2400	2600−3800
k_3 (s^{-1})	340	300−310
k_4 (s^{-1})	1×10^4	1.8×10^4−3.4×10^4
k_{-4} (M^{-1} s^{-1})	1×10^8	1×10^8

[a] Data were taken from ref 7. [b] Constants k_1 and k_{-4} were locked during the fitting using the !ProK global analysis program.

(1) Payne, D. J. *J. Med. Microbiol.* **1993**, *39*, 93−99.
(2) (a) Rasmussen, B. A.; Guzman, Y.; Tally, F. P. *Antimicrob. Agents Chemother.* **1990**, *34*, 1590−1592. (b) Yang, Y.; Rasmuessen, B. A.; Bush, K. *Antimicrob. Agents Chemother.* **1992**, *36*, 1155−1157.
(3) Rasmussen, B. A.; Bush, K.; Tally, F. P. *Clin. Infect. Dis.* **1993**, *16*, S930−S400.
(4) Crowder, M. W.; Wang, Z.; Frankilin, S. L.; Zovinka, E. P.; Benkovic, S. J. *Biochemistry* **1996**, *35*, 12126−12132.
(5) (a) Concha, N. O.; Rasmussen, B. A.; Bush, K.; Herzberg, O. *Structure* **1996**, *4*, 823−836. (b) Concha, N. O.; Rasmussen, B. A.; Bush, K.; Herzberg, O. *Protein Sci.* **1997**, *6*, 2671−2676. (c) Carfi, A.; Duée, E.; Paul-Soto, R.; Galleni, M.; Frère, J.-M.; Dideberg, O. *Acta Crystallogr.* **1998**, *D54*, 47−57.
(6) O'Callaghan, C. H.; Morris, A.; Kirby, S. M.; Shingler, A. H. *Antimicrob. Agents Chemother.* **1972**, *1*, 283−288.
(7) Wang, Z.; Benkovic, S. J. *J. Biol. Chem.* **1998**, *273*, 22402−22408.
(8) Beechem, J. M. *Methods Enzymol.* **1992**, *210*, 37−53.

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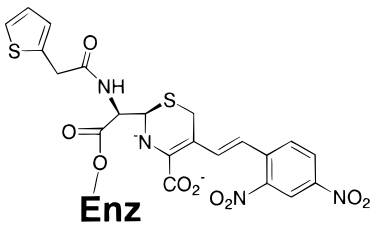

Figure 2. Proposed structure of the acyl enzyme intermediate.

red-shift of the intermediate (to 665 nm) from those of the substrate and product comes as a surprise. Under cryoenzymological conditions, a spectral intermediate, identified as an ES complex, was observed in nitrocefin hydrolysis by the mono-Zn(II) *Bacillus cereus* metallo-β-lactamase, but only showed a modest 48-nm red-shift from that of the substrate.[10] The magnitude of this shift is consistent with that reported for binding to a hydrophobic enzyme pocket[11a] or for *o*-, *m*-, or *p*-nitrostyrenes in solvents of varying polarity[11b] but is much smaller than the shift observed here. Changes in substrate planarity and positioning of charges near the chromophore may also affect the absorbance spectrum, but the most likely cause of the red-shift is an increase in conjugation of the intermediate. The most probable scenario is an enzyme-bound intermediate in which the leaving nitrogen atom is not protonated during the cleavage of the C−N bond and stays negatively charged (Figure 2). This extends the conjugation between the 2,4-dinitrostyryl substituent and the double bond of the dihydrothiazine ring to the negatively charged nitrogen. A similar conjugation occurs when the amino group of 2,4-dinitroaniline is deprotonated by strong base. Indeed, a 178-nm absorbance red-shift (from 336 to 514 nm) is observed upon 2,4-dinitroaniline deprotonation.[12,13] This is comparable to the 175-nm absorbance red-shift (490 to 665 nm) from the hydrolysis product of nitrocefin to the proposed anionic intermediate. Unfortunately, attempts to deprotonate hydrolyzed nitrocefin with strong base did not yield conclusive results because hydrolyzed nitrocefin rapidly decomposes under alkaline conditions.[14]

The nucleophile attacking the Zn(II)-activated carbonyl group of the β-lactam ring during turnover is most likely the deprotonated water shared by both Zn(II) ions in the active site of the enzyme.[5a,c,7] The intermediate observed is a novel acyl intermediate attached to the enzyme through the Zn−O linkage. Earlier kinetic studies carried out in deuterium oxide implicated a solvent isotope effect of ca. 2.6 on the deacylation step.[7] This probably does not reflect protonation of the leaving nitrogen atom, but rather solvent-catalyzed release of the cleaved lactam from the dinuclear Zn(II) center.

How is the anionic intermediate stabilized to such an unusual degree? First, the highly conjugated π orbitals of the 2,4-dinitrostyryl group and the double bond in the dihydrothiazine ring delocalize the electrons of the negatively charged nitrogen. In model studies of amide hydrolysis, reduction of the leaving group amine basicity can destabilize N-protonated forms and facilitate expulsion of the leaving nitrogen without prior protonation.[15] Second, the active site of the enzyme contains two Zn(II) ions.[5] Besides providing an oxyanion hole to polarize the carbonyl group of the β-lactam ring, this histidine-rich Zn(II) environment can provide intense electrostatic stabilization to the negatively charged intermediate and the flanking transition states.[16] It is conceivable that there is strong electrostatic interaction between the negatively charged nitrogen of the intermediate and the second Zn(II) ion, i.e., the Zn(II) with Asp90, Cys168, His210, and the apical water ligands. One proposed docking model of substrates to the enzyme active site places the nitrogen atom of the β-lactam bond in close proximity to the apical water.[5a] The direct interaction of Zn(II) with the negatively charged nitrogen of the cleaved β-lactam bond may exclude the apical water from the Zn(II) ion, thus eliminating a potential proton source. The fact that some Zn(II) ligands such as fluoride and azide accelerate the breakdown of the intermediate further supports this notion.[17] Third, residues from the substrate binding pocket, such as the side chains of Lys171 and Trp36 and the flexible loop of residues 32−38 also provide a favorable environment for a negatively charged species.[18] Detailed structural information about the metallo-β-lactamase with bound transition-state analogues should delineate the myriad forces involved in the stabilization of the intermediate.

In summary, we have demonstrated, by a rapid scanning stopped-flow technique, that the catalytic mechanism of the metallo-β-lactamase from *B. fragilis* involves an acyl enzyme intermediate whose breakdown in turnover is rate-limiting. A novel feature of the mechanism is that the leaving β-lactam nitrogen atom in the bound intermediate is probably not protonated. This is the first example of such an acyl enzyme intermediate in the catalytic cycle of either a metallo-β-lactamase or a Zn(II)-containing protease. Studies to understand how this intermediate is generated, stabilized, and decomposed during catalysis are currently underway.

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Supporting Information Available: Absorbance spectra of the initial (S and ES), intermediate (EI), and final species (EP and P) of the *B. fragilis* metallo-β-lactamase-catalyzed nitrocefin hydrolysis at pH 7.0 and 25 °C (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(9) The hydrolysis product was analyzed with mass spectrometry at the Pennsylvania State University Mass Spectrometry Center. Briefly, the organic compounds (from the reaction mixture of 250 μM nitrocefin and 25 nM enzyme in 100 μL of 1X MTEN, pH 7.0 buffer) was extracted twice with 400 μL of ethyl acetate after adjusting the pH to ca. 4.0 with 1 M HCl. Ethyl acetate was then evaporated by argon flow and the remaining substances were subjected to mass spectrometric analysis in −FAB mode on a KRATOS MS50T mass spectrometer. All of the substances were found to have a molecular weight of 534, indicating that the metallo-β-lactamase-catalyzed nitrocefin hydrolysis was complete and only one product, hydrolyzed nitrocefin, was generated.

(10) Bicknell, R.; Waley, S. G. *Biochemistry* **1985**, *24*, 6876−6887.

(11) (a) Charney, E.; Bernhard, S. A. *J. Am. Chem. Soc.* **1967**, *89*, 2726−2733. (b) Barzaghi, M.; Gamba, A.; Morosi, G.; Simonetta, M. *J. Phys. Chem.* **1974**, *78*, 49−56.

(12) (a) Crampton, M. R.; Gold, V. *J. Chem. Soc. B* **1966**, 893−900. (b) Myakisheva, E. A.; Gershkovich, I. M.; Kaminskii, A. Ya.; Grudtsyn, Y. D. *Zh. Org. Khim.* **1983**, *19*, 664−665.

(13) UV−vis spectra of 2,4-dinitroaniline and its deprotoned product with sodium methoxide in dimethylformamide (DMF) were collected on a Varian Cary-1 spectrophotometer. Small aliquots (less than 2% of total volume) of sodium methoxide in methanol were added to a solution of 2,4-dinitroaniline (53 μM in DMF) until the spectral shift was complete. The starting absorbance peak at 336 nm decreases with concomitant formation of new peaks at 376, 389, and 514 nm.

(14) The UV−vis spectrum of hydrolyzed nitrocefin under strong basic conditions in a variety of organic solvents including acetone, acetonitrile, and ethyl acetate were studied using either a Varian Cary-1 spectrophotometer or an Applied Photophysics SX.18MV stopped-flow spectrometer. In all of the cases, a transient absorbance band between 550 and 600 nm was observed. However, the visible signals became broader and broader and eventually disappeared.

(15) Brown, R. S.; Bennet, A. J.; Slebocka-Tilk, H. *Acc. Chem. Res.* **1992**, *25*, 481−488.

(16) Christianson, D. W.; Fierke, C. A. *Acc. Chem. Res.* **1996**, *29*, 331−339.

(17) Wang, Z.; Fast, W.; Benkovic, S. J., unpublished results.

(18) Toney, J. H.; Fitzgerald, P. M. D.; Grover-Sharma, N.; Olson, S. H.; May, W. J.; Sundelof, J. G.; Vanderwall, D. E.; Cleary, K. A.; Grant, S. K.; Wu, J. K.; Kozarich, J. W.; Pompliano, D. L.; Hammond, G. G. *Chem. Biol.* **1998**, *5*, 185−196.