

Fluorophore-Labeled β -Lactamase as a Biosensor for β -Lactam Antibiotics: A Study of the Biosensing Process

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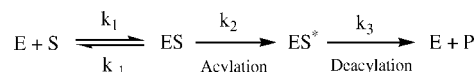
Abstract: The fluorescein-labeled E166C mutant of the PenPC β -lactamase (E166Cf) represents a successful model in the construction of “switch-on” fluorescent biosensors from nonallosteric proteins (Chan P.-H. et al.; *J. Am. Chem. Soc.*, **2004**, 126, 4074). This paper focuses on the study of the biosensing mechanism by which the E166Cf biosensor changes its fluorescence upon β -lactam binding and hydrolysis. Mass spectrometric and stopped-flow fluorescence studies of E166Cf with cefuroxime, penicillin G, and 6-aminopenicillanic acid reveal that the formation of enzyme–substrate complex enhances the fluorescence of E166Cf, and the subsequent regeneration of the free enzyme restores the weak fluorescence of E166Cf. Molecular modeling studies of E166Cf with penicillin G show that the fluorescein label is likely to share a common space with the β -lactam and thiazolidine rings of the antibiotic in the active site. This spatial clash appears to cause the fluorescein label to move from the active site to the external aqueous environment upon substrate binding and hence experience higher water exposure. Steady-state fluorescence measurements indicate that the fluorescence of E166Cf can be enhanced by 6-aminopenicillanic acid, which consists of the β -lactam and thiazolidine rings only. Thermal denaturation experiments of the wild-type enzyme, E166C, and E166Cf reveal that the E166C mutation is likely to increase the flexibility of the Ω -loop. This “modified” structural property might compensate for the possible steric effect of the fluorescein label on substrate binding.

1. Introduction

β -Lactam antibiotics such as penicillins and cephalosporins have been routinely used in the treatment of bacterial infections over the past several decades. These antibacterial agents can inactivate penicillin-binding proteins (PBP), which are responsible for synthesizing bacterial cell walls through the formation of stable covalent acyl complexes.^{1–4} As a consequence of this ‘irreversible’ substrate binding, the cell-wall-synthesizing activity of PBP is strongly inhibited, thus leading to cell death.^{1–4}

The overuse of these drugs, however, has led to the increasing emergence of antibiotic-resistant bacteria, which are able to produce β -lactamase enzymes to destroy β -lactam antibiotics.^{1–4} In this enzyme family, serine β -lactamases from classes A, C,

Scheme 1. Catalytic Pathway of Serine β -Lactamases: where E Is the Enzyme β -Lactamase, S Is an Antibiotic Substrate, ES Is a Non-Covalent Enzyme–Substrate Complex, ES* Is a Covalent Acyl Enzyme–Substrate Complex, and P Is the Product.



and D can efficiently catalyze β -lactam hydrolysis according to a three-step model, which involves the acylation of the enzymes with β -lactam antibiotics (Scheme 1).^{1,2} The subsequent deacylation of the covalent acyl enzyme–substrate complexes leads to the generation of carboxylic acids as products.^{1,2}

One of the effective ways to reduce the emergence of antibiotic-resistant bacteria is to prevent the improper use of β -lactam antibiotics. In this regard, the abuse of β -lactam antibiotics in the food industry (e.g., dairy products) is recognized as a major route of spreading antibiotic resistance in bacteria. To combat this problem, we have recently constructed a “switch-on” fluorescent biosensor (E166Cf) for β -lactam antibiotics from the class A PenPC β -lactamase.⁵ Unlike allosteric ligand-binding proteins which usually undergo

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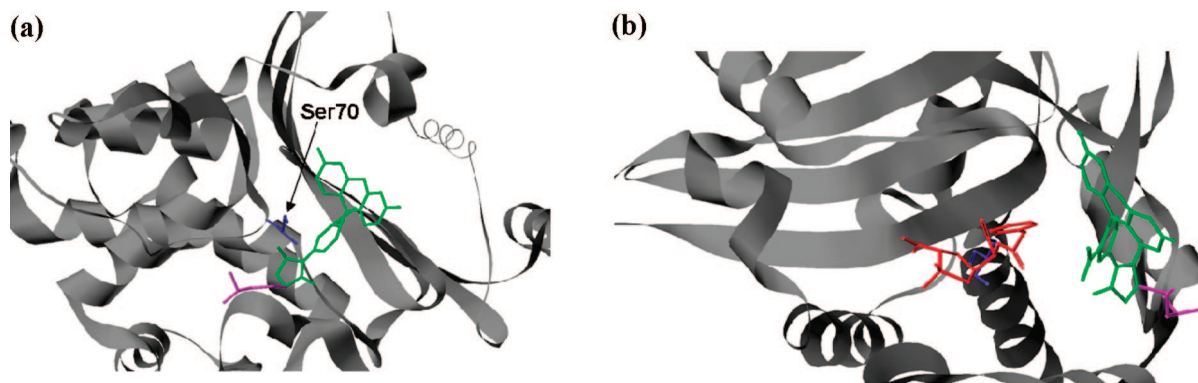


Figure 1. Molecular models of E166Cf with and without penicillin G. (a) Stereoview of the active site of E166Cf without binding to an antibiotic substrate.⁵ The fluorescein label (green) is buried inside the active site in the substrate-free E state. (b) Stereoview of the active site of E166Cf in the covalent ES* state.⁵ Note that the fluorescein label (green) stays out of the active site to avoid the spatial clash with the penicillin G molecule (red) in this state. The Ser70 and Cys166 residues are shown in blue and magenta, respectively.

large conformational changes upon ligand binding,^{6–16} this bacterial enzyme is nonallosteric, and therefore, detecting the substrate binding in the enzyme's active site requires the placement of a fluorophore close to this local environment. The catalytically important residue Glu166 in the flexible Ω -loop (close to the active site)^{17–20} of the wild-type enzyme was first replaced with a cysteine, which was subsequently labeled with the environment-sensitive fluorophore fluorescein-5-maleimide.⁵ The removal of the catalytic residue significantly reduces the hydrolytic activity of E166Cf, thus allowing E166Cf to serve as a “ligand-binding” protein (with very low k_{cat}).⁵ The E166Cf mutant fluoresces more strongly upon binding to penicillins (e.g., penicillin G and ampicillin) and cephalosporins (e.g., cefuroxime and cefoxitin). For the penicillin antibiotics, the E166C mutant can still hydrolyze these substrates, albeit slowly, and gives declining fluorescence signals.⁵ In contrast, the E166Cf mutant gives stronger fluorescence with the cephalosporin antibiotics over a sustained period of time due to their strong resistance to the hydrolytic activity of the enzyme. Molecular modeling studies of E166Cf have shown that the fluorescein label moves out of the active site upon binding to an antibiotic (Figure 1).⁵ This subtle motion causes the fluorescein label to gain higher

water exposure and hence give stronger fluorescence.⁵ Surprisingly, the attached fluorophore does not significantly impair the E166Cf–antibiotic binding, presumably due to the flexible nature of the Ω -loop.^{17–20}

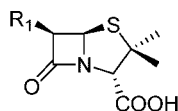
The E166Cf mutant represents a successful case in the construction of fluorescent biosensors from nonallosteric proteins. Understanding the biosensing mechanism of E166Cf is of particular importance because this can provide an insight into the way of constructing “switch-on” fluorescent biosensors for biologically significant substrates/ligands from nonallosteric proteins in general. This biosensor development will advance the analytical science in a variety of important fields, ranging from clinical diagnosis to environmental monitoring.

In this study, we further investigated the biosensing mechanism of the E166Cf mutant, using a variety of biophysical techniques including electrospray ionization mass spectrometry (ESI-MS), stopped-flow fluorescence spectroscopy, and circular dichroism (CD) spectropolarimetry. Parallel mass spectrometric and stopped-flow fluorescence measurements show that the formation of enzyme–substrate complex causes E166Cf to enhance its fluorescence. The subsequent hydrolysis of the bound substrate causes E166Cf to restore its weak fluorescence signal. In addition, molecular modeling studies reveal that the fluorescein label is likely to share a common space within the active site with the bicyclic structure of β -lactam antibiotics. This spatial clash appears to cause the fluorescein label to depart from the active site upon substrate binding and gain higher exposure to the external aqueous environment. This observation is further supported by the findings of the proteolytic studies of E166Cf. In addition, thermal unfolding experiments monitored by CD spectropolarimetry reveal that the Ω -loop of E166Cf, where the fluorescein label resides, gains higher flexibility compared to that of the wild-type enzyme.

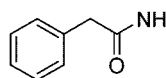
2. Experimental Section

2.1. Chemicals. Penicillin G, cefuroxime, 6-aminopenicillanic acid (6-APA), chloramphenicol, potassium dihydrogenphosphate, guanidine hydrochloride (GdnHCl), sodium chloride, TrisHCl, ammonium bicarbonate, ammonium acetate, trypsin from bovine pancreas, and myoglobin from horse heart were obtained from Sigma Co. Fluorescein was purchased from Fluka. Fluorescein-5-maleimide was obtained from Molecular Probes, Inc. The structures of the antibiotics and the fluorophore used in this study are shown in Figure 2.

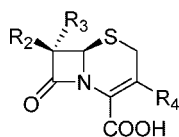
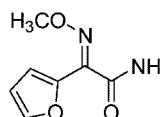
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PenicillinsR₁NH₂

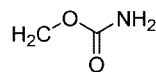
6-Aminopenicillanic acid (6-APA)



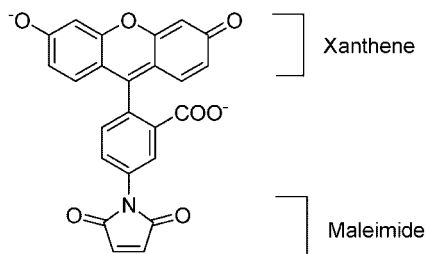
Penicillin G

CephalosporinR₂R₃R₄

H



Cefuroxime

Fluorescein-5-maleimide

Xanthene

Maleimide

Figure 2. Structures of the β -lactam antibiotics and fluorescein-5-maleimide used in this study.

2.2. Protein Preparation and Characterization. The wild-type PenPC β -lactamase was expressed and purified as described.⁵ The E166C mutant of the PenPC β -lactamase was expressed by fermentation and purified with Celite 545 (see Supporting Information). The fluorophore labeling of E166C was performed according to the method described in our previous studies.⁵ The mass values of the wild-type and mutant enzymes (E166C and E166Cf) were analyzed by ESI-MS.⁵ The kinetic parameters of the wild-type and mutant enzymes were determined by the spectrophotometric method⁵ in which the hydrolysis of penicillin G, 6-APA and cefuroxime were monitored at 232, 240 and 260 nm, respectively. Steady-state fluorescence measurements of the E166Cf mutant with penicillin G, 6-APA and cefuroxime were performed as described.⁵ The experimental details for molecular modeling and mathematical simulation are described in Supporting Information.

2.3. Enzyme Kinetics Studies by Mass Spectrometry.

2.3.1. Sample Preparation. The E166C and E166Cf samples were further purified prior to mass spectrometric measurements as follows. The protein samples in phosphate buffer (pH 7.2) were loaded onto a CM column (HiTrap CM Sepharose FF, 1 mL, GE

Healthcare), which was pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.2). The proteins were eluted by a linear gradient of NaCl (0–0.5 M). The desired fractions were collected, concentrated, and buffer-exchanged with 20 mM ammonium acetate (pH 7.0) for at least five cycles by means of Amicon Ultra-15 (NMWL = 10,000) centrifugal filter devices (Millipore).

2.4. Detection of Covalent Acyl Enzyme–Substrate Complexes.

Covalent acyl enzyme–substrate complexes formed by β -lactamases and PBP can be detected by ESI-MS^{21–24} and fluorescence spectroscopy.^{25,26} In this study, we studied the catalytic processes of the mutant enzymes with cefuroxime and 6-APA by the mass spectrometric method.^{21–24} The enzyme–substrate binding reaction was initiated by mixing 60 μ L of 1 μ M E166Cf (in 20 mM ammonium acetate buffer, pH 7.0) with 60 μ L of 10 μ M antibiotic (cefuroxime and 6-APA, in 20 mM ammonium acetate buffer, pH 7.0). The reaction was allowed to take place at room temperature. At desired time intervals, the reaction was quenched by adding 120 μ L of 8% formic acid in CH₃CN to the enzyme solution (to unfold the protein), giving a reaction mixture in buffer/CH₃CN (1:1 v/v) containing 4% formic acid (pH $\approx$ 2). For short reaction times, the reaction was initiated and quenched by a quench-flow system (Biologic SFM-400/Q, Claix, France). All quenched samples were stored on ice prior to mass spectrometric analysis. The resulting ESI spectrum was found to show two major peaks (A and B) attributed to the free enzyme E and the enzyme–substrate complex ES*, respectively (refer to Figure S1 and Figure 4). The relative amount of ES* can be expressed as the ratio of the amount of ES* to the total amount of the enzyme, [ES*]/[E_{total}], where [ES*] is the peak area of peak B and [E_{total}] (= [E] + [ES*]) is the sum of peak areas of peak A and B in the transformed ESI mass spectrum. Similar experiments were also performed with the unlabeled E166C mutant. For comparison, parallel stopped-flow fluorescence measurements were also performed with E166Cf (0.5 μ M) with 5 μ M cefuroxime, 6-APA and penicillin G in 20 mM ammonium acetate (pH 7.0), using a stopped-flow instrument equipped with a fluorescence readout device (Applied Photophysics SX.18MV-R, Leatherhead, UK). The sample was excited at 494 nm and the fluorescence signal monitored at 515 nm. Both excitation and emission slit widths were 5 nm.

2.5. Determination of Kinetic Parameters. The methodology described by Lu et al. for studying PBP, which shows mechanistic and kinetic properties similar to those of class A β -lactamases in the binding of β -lactam antibiotics,^{23,24} was adopted in this study. According to Scheme 1, K_d (= k_{-1}/k_1) is the equilibrium dissociation constant for the formation of the reversible noncovalent ES complex, k_2 is the rate constant for the formation of the covalent acyl ES* complex, and k_3 is the rate constant for the hydrolysis of ES* to the free enzyme (deacylation). Taking into consideration that (i) the k_3 values for the Glu166-substituted mutants of the class A TEM-1 and *S. albus* G β -lactamases with β -lactam antibiotics are much lower than those of the wild-type enzymes ($\sim$ 5–9 orders of magnitude lower),²⁷ (ii) the acylation step remains to be active in the Glu166-substituted β -lactamase mutants with β -lactam antibiotics, as reflected by their relatively high acylation efficiency (k_2/K_d),²⁷ and (iii) the predicted k_2 value for the E166D mutant of the PenPC β -lactamase is $\sim$ 2 s^{−1},²⁸ which is much larger than the

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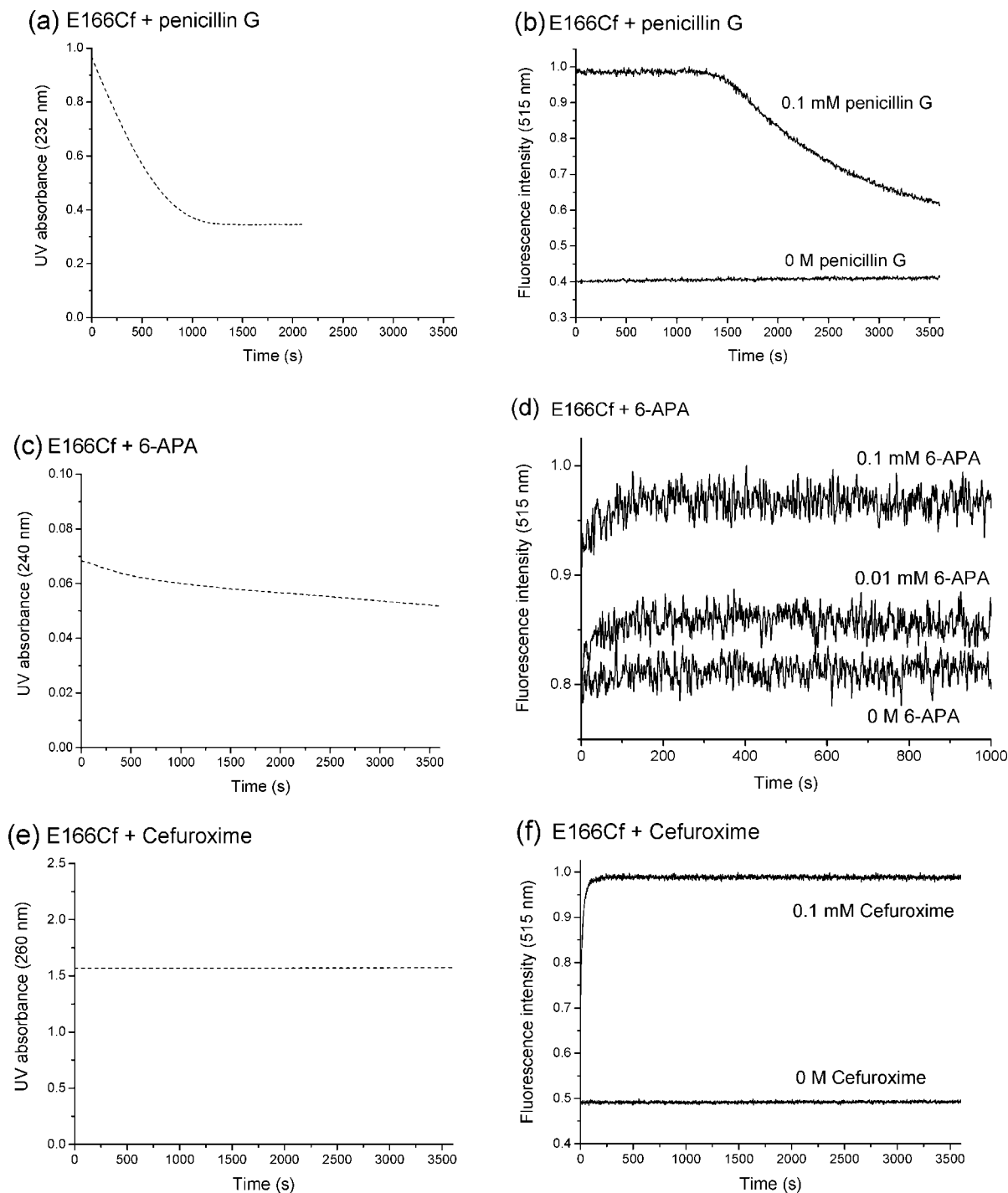


Figure 3. Time course of the hydrolysis of penicillin G, 6-APA, and cefuroxime by E166Cf monitored by UV absorption and fluorescence measurements. The reaction was initiated by mixing E166Cf with each of the antibiotics in 50 mM potassium phosphate (pH 7.0) in both experiments. The UV absorbances of penicillin G, 6-APA, and cefuroxime (0.1 mM) in the presence of E166Cf (0.12 μ M) are shown in (a), (c), and (e), respectively. The decreases in absorbance at 232, 240, and 260 nm arise from the cleavage of the β -lactam amide bond of penicillin G, 6-APA, and cefuroxime, respectively. The experimental data for penicillin G are from ref 5. The fluorescence signals of E166Cf (0.12 μ M) with and without penicillin G, 6-APA and cefuroxime are shown in (b), (d), and (f), respectively. Excitation wavelength: 494 nm; excitation and emission slit widths: 5 nm.

k_3 value of the TEM-1 E166N mutant ($\sim 10^{-6} \text{ s}^{-1}$),²⁷ it is reasonable to assume that the deacylation step is significantly slower than the acylation step. That is, the contribution of deacylation to the formation of ES* is negligible. Thus, the formation of ES* for E166Cf (or E166C) can be regarded as a pseudo-single-turnover process, as in the case of PBP.^{23,24} In addition, the deacylation rate k_3 for the TEM-1 E166N mutant²⁷ is in the same order of magnitude as those of several PBPs,^{23,24} further indicating the similarity in the kinetic properties of Glu166-substituted class A

β -lactamase and PBP. Thus, the kinetic equation for the formation of ES* is given by eq 1:

$$[\text{ES}^*]/[\text{E}_{\text{total}}] = 1 - \exp(-k_a t) \quad (1)$$

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k_a , the apparent first-order rate constant for the formation of ES^* , was obtained by computer-fitting the experimental value of $[ES^*]/[E_{total}]$ versus time (t) to equation [1].

For the mechanism shown in Scheme 1, k_a is related to K_d and k_2 according to equation [2]:

$$k_a = k_2 \frac{[S_0]}{K_d + [S_0]} \quad (2)$$

where $[S_0]$ is the initial substrate concentration and k_2 and K_d were obtained by computer fitting the experimental data of k_a versus $[S_0]$ to equation [2].

The first-order deacylation rate constant k_3 was obtained by monitoring the degradation of ES^* as a function of time.

$$[ES^*]/[E_{total}] = 1 - \exp(-(k_3 t)) \quad (3)$$

The k_3 values were obtained by fitting the experimental data of $[ES^*]/[E_{total}]$ versus time to eq 3. All curve fittings were performed with Origin 6.1.

2.6. Determination of K_d and k_2 . The kinetics studies of E166Cf with cefuroxime and 6-APA were performed using a quench-flow device (Biologic SFM-400/Q, Claix, France). The hydrolytic reaction was initiated by mixing 72.5 μ L of 1 μ M E166Cf (or E166C) in 20 mM ammonium acetate buffer (pH = 7.0) with 72.5 μ L of desired concentrations of antibiotic (cefuroxime and 6-APA) in 20 mM ammonium acetate buffer (pH = 7.0), and then quenched at various time intervals by addition of 145 μ L of 8% (v/v) formic acid. The final pH of the quenched solution was $\sim$ 2. The reaction times were computer-controlled by varying the flow rate of the reactants. The quenched reaction mixtures were injected into the ESI source of the Q-TOF 2 mass spectrometer (Waters-Micromass, Manchester, UK). The mass spectrometer was scanned over a m/z range of 700–1600, and the raw spectra were deconvoluted by the MassLynx 3.5 Transform Program (Waters, Manchester, UK). The ratio of the concentration of the covalent enzyme–substrate complex to the total enzyme concentration ($[ES^*]/[E_{total}]$) at different time intervals was determined by measuring the peak areas of the $[E]$ and $[ES^*]$ peaks in the mass spectrum, where $[E_{total}] = [E] + [ES^*]$. The experimental profile of $[ES^*]/[E_{total}]$ versus time was fitted into eq 1, from which the first order apparent rate constant k_a was obtained. The k_a values at several different substrate concentrations, $[S_0]$, were then determined. The K_d and k_2 values were obtained by fitting the data of k_a versus $[S_0]$ into eq 2.

Similar to the kinetics study by Lu et al. on PBP, the present mass spectrometric study on the kinetics of the β -lactamase mutants was also based on the “rapid-equilibrium assumption” (i.e., $k_2 \ll k_{-1}$), such that K_d could be regarded as an equilibrium dissociation constant for the noncovalent ES complex. In our case, with the relatively large K_d ($\sim$ 1 mM) and small k_2 ($\sim$ 2 s $^{-1}$) values, and the fact that the reported k_1 values for protein–ligand interactions in the literature are in the range of 10^5 – 10^8 M $^{-1}$ s $^{-1}$,²⁴ the estimated k_{-1} value is 10^2 – 10^4 -fold larger than the k_2 value. This argues that the kinetic properties of our E166C mutant are in agreement with the “rapid-equilibrium” assumption,^{23,24} which is equally applicable to the case of the E166Cf biosensor.

2.7. Determination of k_3 . A 500 μ L portion of 1 μ M E166Cf (or E166C) was allowed to react with 500 μ L of 1 μ M antibiotic (cefuroxime and 6-APA) long enough until most of the mutant enzyme was converted to the covalent acyl enzyme–substrate complex, and the deacylation rate constant k_3 was determined by monitoring the dissociation of ES^* with time by ESI-MS. At different sampling points, a 70 μ L portion of the reaction mixture was taken out and quenched with formic acid (final concentration: 4% (v/v)). The ratios of the concentration of the covalent enzyme–substrate complex to the total enzyme concentration, $[ES^*]/[E_{total}]$, at different time intervals were determined as described, and the experimental data were fitted into eq 3, from which the k_3 value was determined.

The kinetic data for E166C and E166Cf so obtained are presented in Figures S1–S7 (Supporting Information) and Figure 4.

2.8. Proteolytic Studies. 2.8.1. Fluorescence Measurements.

E166Cf (0.2 mg/mL) was mixed with trypsin (0.01 mg/mL) in 50 mM potassium phosphate buffer (pH 7.0) in a quartz cuvette of 1 cm path length. The fluorescence spectra of this sample were then recorded at various time intervals using a Perkin-Elmer LS50B spectrofluorimeter. The excitation wavelength was 460 nm, and the excitation and emission slit widths were 2.5 nm.

2.8.2. Mass Spectrometric Measurements. Proteolysis of E166Cf (or E166C, $\sim$ 0.3 mg/mL) was performed by incubation with trypsin ($\sim$ 0.01 mg/mL) in 20 mM ammonium acetate (pH 7.0) at 37 $^{\circ}$ C overnight. The digested sample was mixed with the MALDI matrix composed of 10 mg/mL CHCA in acetonitrile/ethanol/0.1% trifluoroacetic acid (49.5: 49.5: 1 (v/v)). A 1.5 μ L portion of the sample/matrix mixture was spotted onto a 96-well stainless steel target plate, air-dried, and then analyzed by a Waters MicroMX MALDI-TOF mass spectrometer (Waters, Manchester, UK) controlled by MassLynx 4.1 (Waters, Manchester, UK). Peptide ions were generated by a 337 nm pulse laser and accelerated by a voltage of 1.9 kV with a pulse delay of 500 ns. The resulting mass spectra were obtained by accumulation of at least 50 single scans. The peptide fragments of trypsin were identified by accurate mass measurements as follows. A m/z range of 700–3000 Da was calibrated externally with a polyethylene glycol (PEG) mixture (PEG600/PEG1000/PEG2000/NaI = 1:1:3:1 (w/w)). The accurate mass values of the peptide fragments of trypsin were then determined, using a phosphorylase B tryptic digest standard as the internal standard for internal lock mass calibration to achieve a mass accuracy of <20 ppm. The mass peaks of the individual protein fragments were assigned, using the Findpept Program at <http://us.expasy.org/tools/findpept.html>.

2.8.3. Circular Dichroism Measurements. Digested E166Cf was prepared as described above (section 2.8.2.). The far-UV CD spectrum of this sample was recorded on a JASCO-J810 CD spectropolarimeter at a scan rate of 50 nm/min and a bandwidth of 2 nm, using a quartz cuvette of 1 mm path length. For comparison, similar CD measurements were also performed with E166Cf ($\sim$ 0.3 mg/mL) without subsection to trypsin digestion.

2.9. Thermal Unfolding Studies. The thermal unfolding studies of the wild-type PenPC β -lactamase, E166C, and E166Cf in 50 mM potassium phosphate buffer (pH 7.0) were performed on a JASCO J-810 CD spectropolarimeter. The far-UV CD signal at 222 nm was recorded every 1 $^{\circ}$ C from 20 to 80 $^{\circ}$ C for the wild-type enzyme and from 20 to 70 $^{\circ}$ C for E166C and E166Cf, using a quartz cuvette of 1 mm path length. The heating rate and the slit width were 30 $^{\circ}$ C/h and 2 nm (respectively), and the response time was 2 s. The enzymes unfolded reversibly upon thermal denaturation. The CD signals of the enzymes were subtracted from those contributed by the buffer.

The thermal unfolding curves of the enzymes were then fitted to the following equation to determine the midpoints of transitions based on a two-state model for the transition between the native and unfolded states of a protein:²⁷

$$y_{obs} = \frac{\{y_N + pT\} + \{y_U + qT\} \exp[b]}{1 + \exp[b]}$$

where $b = [\Delta H_m(1 - T/T_m)]/RT$, y_{obs} is the measured variable parameter at a given temperature, y_N and y_U are the variable parameters for the native and denatured states (respectively), T_m is the midpoint of the heat-induced transition, ΔH_m is the enthalpy change for unfolding at T_m , p and q are the slopes of the pre- and post-unfolding baselines (respectively), R is the gas constant, and T is the absolute temperature.

3. Results

3.1. Steady-State Fluorescence and Spectrophotometric Studies.

The origin of the fluorescence changes of E166Cf in the presence of penicillin G, 6-APA, and cefuroxime was investigated by both UV absorption and fluorescence measurements.

Cefuroxime is resistant to the hydrolytic action of E166Cf, whereas penicillin G is not.⁵ These antibiotics are good candidates for studying the fluorescence behavior of E166Cf. For 6-APA, this antibiotic is structurally similar to penicillin G except that it has no R₁ chain (Figure 2). Thus, the effect of the bicyclic structure of the β -lactam and thiazolidine rings on the fluorescence response of E166Cf can be examined.

Figure 3a shows the real-time UV absorbance of E166Cf (0.12 μ M) with penicillin G (0.1 mM) in 50 mM phosphate buffer (pH 7.0).⁵ The UV absorption signal at 232 nm, which arises from the UV absorption by the β -lactam amide bond of penicillin G,²⁹ decreases rapidly within 20 min as a result of the hydrolytic action of E166Cf toward the β -lactam ring. After 20 min of β -lactam hydrolysis, the UV absorption signal becomes steady. Interestingly, under similar conditions, E166Cf exhibits a sustained fluorescence plateau for about 20 min in the presence of the same concentration of penicillin G (Figure 3b).⁵ This fluorescence change, however, was not observed when the unlabeled E166C mutant was incubated with free fluorescein under similar conditions (Figure S8), indicating that the fluorescence change observed with E166Cf is likely to arise from the local environmental change around the fluorescein label induced by antibiotic binding. Taking these observations together, the fluorescence enhancement of E166Cf is likely to be triggered by the binding of penicillin G to the active site. It is interesting to note that the fluorescence of E166Cf declines slowly after the hydrolysis of penicillin G ($t > 20$ min). This observation is presumably due to the slow return of the fluorescein label to the vacant active site after β -lactam hydrolysis because of its bulky nature.

Unlike penicillin G, 6-APA shows only a slight decrease in UV absorbance over the time course, indicating that the hydrolysis of 6-APA by E166Cf is very slow (Figure 3c). In addition, fluorescence measurements showed that the fluorescence of E166Cf with 6-APA increases as a function of time (Figure 3d). This increasing fluorescence profile is similar to the fluorescence/time profiles corresponding to the initial phase of hydrolysis of penicillin and cephalosporin antibiotics.⁵ Unlike the case of penicillin G,⁵ the fluorescence enhancement for 6-APA is much less significant, and no detectable fluorescence decline appears over the time course. These observations are presumably due to the weaker binding and activity of E166Cf toward 6-APA compared to that toward penicillin G.

For cefuroxime, this antibiotic shows no significant decrease in UV absorbance, indicating its high resistance to the hydrolytic action of E166Cf (Figure 3e). Moreover, E166Cf shows a sustained fluorescence plateau over the time course, similar to the case of 6-APA (Figure 3f). These results, together with those obtained with penicillin G, highlight the significant role of β -lactam binding in enhancing the fluorescence of E166Cf.

3.2. Mass Spectrometric and Stopped-Flow Fluorescence Studies. In order to further investigate the effects of antibiotic binding and hydrolysis on the fluorescence of E166Cf, the hydrolytic processes of cefuroxime, 6-APA, and penicillin G by E166Cf at different time intervals were analyzed by ESI-MS. This technique can distinguish ES* from E according to their different mass values and determine the population of ES* over the time course. Briefly, E166Cf was mixed with each of the antibiotics, and the hydrolytic process was quenched by acid-unfolding of the labeled enzyme at different time intervals. The

population of the covalent acyl enzyme–substrate complex ES* at each time interval was then determined (see Experimental Section).

As shown in Figure 4e, the population of the ES* state for cefuroxime increases slowly as a function of time (up to 15 min), indicating that this antibiotic is resistant to the acylating action of E166Cf. For 6-APA, the population of ES* also increases over the time course (Figure 4f). In both cases, no significant decline in the ES* population was observed over the time course, indicating that cefuroxime and 6-APA are resistant to the deacylating action of E166Cf. In the case of penicillin G, the population of ES* increases rapidly in the first 60 s and then declines gradually (Figure 4g). The decrease in the ES* population indicates that penicillin G is less resistant to the deacylating action of E166Cf compared to cefuroxime and 6-APA.

Similar mass spectrometric experiments were also performed with the unlabeled E166C mutant, and the kinetic parameters for E166C and E166Cf were then analyzed (see Experimental Section). As similar to the case of E166Cf, the ES* state of E166C with cefuroxime accumulates over the time course, indicating that this antibiotic is also resistant to the acylating action of E166C (Figure S3). Detailed analysis of the mass spectral data for E166C and E166Cf showed that the k_{cat} values of both mutant enzymes with cefuroxime are similar, implying that the fluorescein label does not significantly impair the enzymatic activity (Table 1). The k_{cat} values for E166C and E166Cf with cefuroxime are lower than that of the wild-type enzyme (~ 100 -fold lower, Table 1), an observation consistent with the fact that Glu166 is important for β -lactam hydrolysis in class A β -lactamases.^{28,30} Moreover, the K_d value of E166C is similar to that of E166Cf, indicating that the fluorescein label does not significantly impair the substrate binding ability of the mutant enzyme.

For 6-APA, the ES* populations of E166Cf and E166C also increase over the time course (Figures S5 and S6). The k_{cat} value of E166Cf is similar to that of E166C but much lower than that of the wild-type enzyme, as similar to the case of cefuroxime (Table 1). Moreover, the k_{cat} value for 6-APA is similar to that for cefuroxime but significantly lower than that for penicillin G (~ 4 orders of magnitude lower), indicating that 6-APA and cefuroxime are more resistant to the hydrolytic action of E166Cf compared to penicillin G. This observation is consistent with the findings of the spectrophotometric experiments that the decrease in UV absorbance of penicillin G with E166Cf is much more significant than those of cefuroxime and 6-APA (Figure 3).

We then performed stopped-flow fluorescence measurements on E166Cf with each of the antibiotics under similar conditions. As shown in Figure 4e, the fluorescence signal of E166Cf increases as a function of time upon addition of cefuroxime. Interestingly, this time-dependent fluorescence profile matches closely with the mass spectral [ES*]/time profile (Figure 4e), implying that the fluorescence enhancement is likely to arise from substrate binding. Similar observations were also obtained with 6-APA (Figure 4f). For penicillin G, the fluorescence of E166Cf increases rapidly in the early stage of the time course and then declines slowly (Figure 4g). This fluorescence profile also matches closely with the mass spectral [ES*]-time profile. All these observations indicate that the fluorescence enhance-

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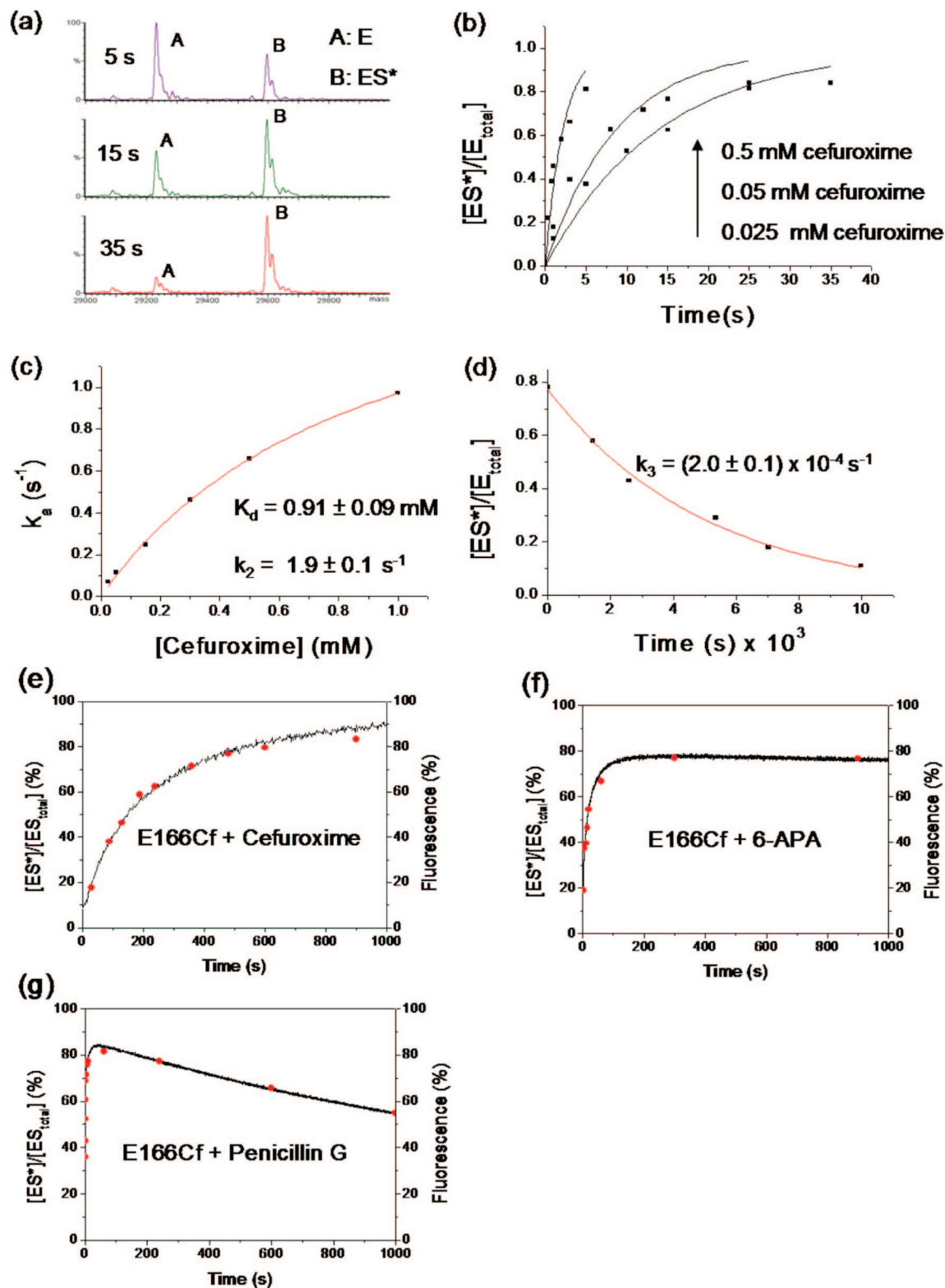


Figure 4. Mass spectrometric and stopped-flow fluorescence studies of E166Cf binding with cefuroxime, 6-APA, and penicillin G. (a) Transformed mass spectra acquired after incubating E166Cf with 0.1 mM cefuroxime at different time intervals. (b) Time course for the acylation of E166Cf with different concentrations of cefuroxime. The solid lines represent the fit of experimental data to eq 1, from which the k_a values were obtained. (c) Plot of the k_a values as a function of cefuroxime concentrations. The solid line represents the fit of experimental data to eq 2, from which the K_d and k_2 values were found to be 0.91 ± 0.09 mM and 1.9 ± 0.1 s^{-1} , respectively. (d) Time course for the deacylation of the E166Cf–cefuroxime complex. The solid line represents the fit of experimental data to eq 3, from which the k_3 value was determined. (e–g) Time course for the binding of E166Cf (0.5 μ M) with 5 μ M cefuroxime, 6-APA, and penicillin G (respectively) monitored by ESI-MS and stopped-flow fluorescence spectroscopy. The solid lines represent the fluorescence signals, and the red circles represent the $[ES^*]/[E_{total}]$ values of the E166Cf–antibiotic complexes determined by ESI-MS. Buffer: 20 mM ammonium acetate (pH 7.0).

Table 1. Kinetic Parameters of the Wild-Type PenPC β -Lactamase, E166C and E166Cf for the Hydrolysis of Cefuroxime, Penicillin G, and 6-APA Determined by ESI-MS and UV–Visible Spectrophotometry^a

	K_m (μ M)	K_d (mM)	k_2 (s^{-1})	k_2/K_d (k_{cat}/K_m) ($M^{-1} s^{-1}$)	k_3 (s^{-1})	k_{cat} (s^{-1}) ^d
wild-type + cefuroxime ^b	85 $\pm$ 4	—	—	(0.2 $\pm$ 0.1) $\times 10^3$	—	0.02 $\pm$ 0.01
E166C + cefuroxime ^c	—	2.9 $\pm$ 0.3	2.5 $\pm$ 0.2	(0.9 $\pm$ 0.1) $\times 10^3$	(7.0 $\pm$ 0.3) $\times 10^{-5}$	(7.2 $\pm$ 0.9) $\times 10^{-5}$
E166Cf + cefuroxime ^c	—	0.91 $\pm$ 0.09	1.9 $\pm$ 0.1	(2.0 $\pm$ 0.2) $\times 10^3$	(2.0 $\pm$ 0.1) $\times 10^{-4}$	(2.0 $\pm$ 0.2) $\times 10^{-4}$
wild-type + penicillin G, ^e	48 $\pm$ 3	—	—	(5.4 $\pm$ 0.1) $\times 10^7$	—	2612 $\pm$ 320
E166C + penicillin G, ^e	72 $\pm$ 3	—	—	(2.9 $\pm$ 0.2) $\times 10^4$	—	2.07 $\pm$ 0.02
E166Cf + penicillin G, ^e	213 $\pm$ 11	—	—	(2.5 $\pm$ 0.2) $\times 10^4$	—	5.28 $\pm$ 0.09
wild-type + 6-APA ^b	1374 $\pm$ 42	—	—	(5.5 $\pm$ 0.2) $\times 10^5$	—	760 $\pm$ 10
E166C + 6-APA ^c	—	0.23 $\pm$ 0.02	16.7 $\pm$ 0.7	(7.3 $\pm$ 0.7) $\times 10^4$	(2.4 $\pm$ 0.4) $\times 10^{-3}$	(2.4 $\pm$ 0.4) $\times 10^{-3}$
E166Cf + 6-APA ^c	—	0.7 $\pm$ 0.1	0.28 $\pm$ 0.02	(4.0 $\pm$ 0.6) $\times 10^2$	(8.0 $\pm$ 1.0) $\times 10^{-4}$	(8.0 $\pm$ 1.0) $\times 10^{-4}$

^a ESI-MS was used to study the enzyme kinetics in which the catalytic process could not be monitored by the spectrophotometric method (e.g., E166C/cefuroxime, E166C/6-APA, E166Cf/cefuroxime, and E166Cf/6-APA). For the wild-type enzyme, the kinetics was so fast that the MS method could not determine the catalytic parameters for the antibiotics. This is also the case for the reaction of the wild-type and mutant enzymes with penicillin G. ^b Monitored by UV–visible spectrophotometry. ^c Monitored by ESI-MS. ^d $k_{cat} = k_2k_3/k_2 + k_3$. ^e Reference 5; — not determined.

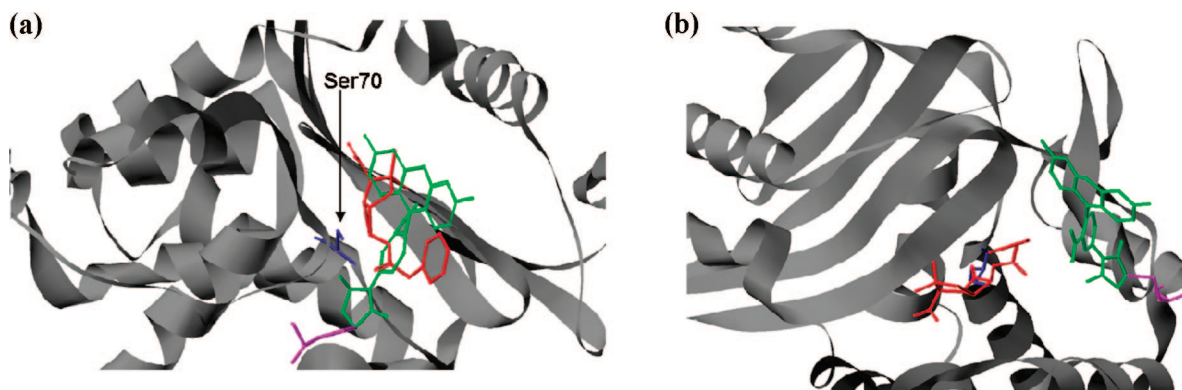


Figure 5. Molecular models of E166Cf with penicillin G. (a) Stereoview of the active site of E166Cf (with the fluorescein label staying inside the active site) docked with an intact penicillin G molecule. Note that the xanthene ring and the benzoic group of the fluorescein label (green) clash spatially with the thiazolidine ring and the exocyclic R_1 amide group of the penicillin G molecule (red), respectively. (b) Stereoview of the active site of E166Cf in the noncovalent ES state. The fluorescein label (green) stays out of the active site to avoid the spatial clash with the penicillin G molecule (red) in this state. The Ser70 and Cys166 residues are shown in blue and magenta, respectively.

ment of E166Cf arises from the formation of enzyme–substrate complex, whereas the subsequent regeneration of the free enzyme restores the weak fluorescence of E166Cf.

3.3. Molecular Modeling. In order to investigate the mechanism by which E166Cf enhances its fluorescence upon binding to β -lactam antibiotics, molecular modeling was performed with this mutant enzyme in the presence and absence of penicillin G. This antibiotic was chosen because it can bind to E166Cf and the crystal structures of various class A β -lactamases complexed with this drug have been extensively studied.^{31–33} Figure 1a shows the stereoview of the active site of E166Cf in the free enzyme state.⁵ The protein model shows that both the side chain of Cys166 in the Ω -loop and the attached fluorescein label are oriented toward the active site. The fluorescein label is buried in the active site with a solvent accessible area (SAA) of 191 $\text{\AA}^2$. In order to examine whether the fluorescein label shares a common space with the β -lactam substrate within the active site, the structure of E166Cf (with the fluorescein label staying inside the active site) was docked with an intact penicillin G molecule (Figure 5a). Detailed analysis of this structure showed that both the fluorescein label and the substrate share the same space within the active site; the xanthene ring and the benzoic group (above the maleimide linker) of the

fluorescein label clash spatially with the thiazolidine ring and the exocyclic R_1 amide group of the antibiotic, respectively (see Figures 2 and 5a). In the ES state, the fluorescein label stays out of the active site (Figure 5b). This subtle conformational change results in a significant increase in water exposure for the fluorescein label in the ES state (SAA = 350 $\text{\AA}^2$) compared to that of the E state (SAA = 191 $\text{\AA}^2$). Similar observations were also obtained with the covalent ES* state in which the hydroxyl group of the side chain of Ser70 is acylated with the β -lactam carbonyl group of the substrate;^{1–3} the fluorescein label resides in the external aqueous environment with a SAA of 350 $\text{\AA}^2$ after the covalent binding of the substrate to the active site (Figure 1b).⁵

The steric effect of the bicyclic structure of β -lactam antibiotics on the positioning of the fluorescein label is further supported by the fluorescence experiments on 6-APA (consisting of the β -lactam and thiazolidine rings only (i.e., no R_1 chain, Figure 2)); the fluorescence signal of E166Cf with 6-APA increases over the time course (Figure 3(d)). This observation also appears in the fluorescence-time profiles corresponding to the initial phase of hydrolysis of penicillin and cephalosporin antibiotics.⁵

3.4. Proteolytic Studies. The water exposure change induced by antibiotic binding can be probed by investigating the fluorescence response of the fluorescein label (whose emission

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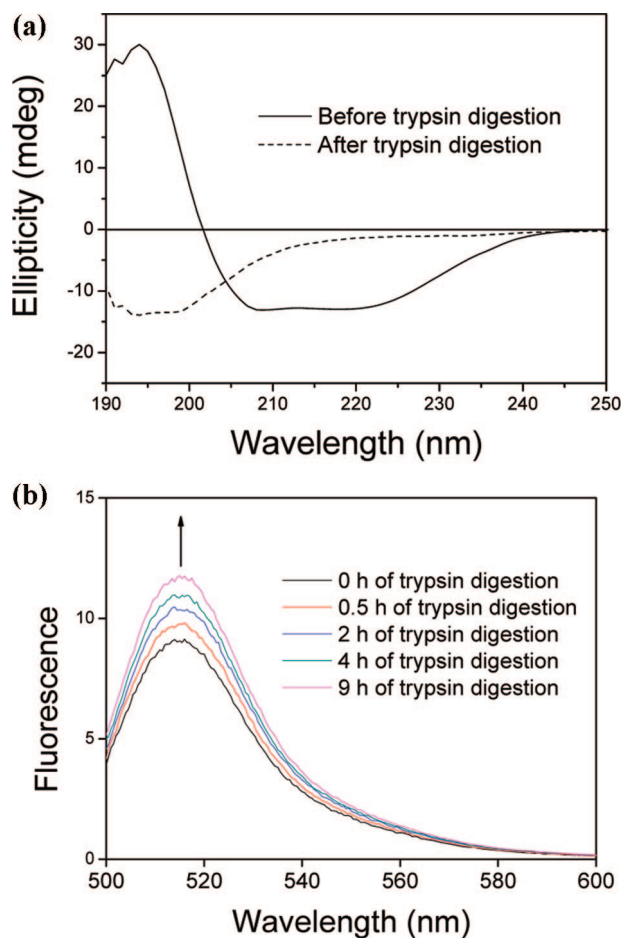


Figure 6. Proteolytic studies of E166Cf analyzed by CD spectropolarimetry and fluorescence spectroscopy. (a) Far-UV CD spectra of E166Cf (~ 0.3 mg/mL) before (solid line) and after (dash line) trypsin digestion in 20 mM ammonium acetate (pH 7.0). The CD signals were recorded at a scan rate of 50 nm/min and a bandwidth of 2 nm, using a quartz cell of 1 mm path length. The concentration of trypsin was 0.01 mg/mL. (b) Fluorescence spectra of E166Cf in the presence of trypsin recorded at different time intervals. The concentrations of E166Cf and trypsin were 0.2 mg/mL and 0.01 mg/mL, respectively. Excitation wavelength: 460 nm; excitation and emission slit widths: 2.5 nm.

is solvent-sensitive)^{34,35} before and after proteolytic digestion, since this fluorophore is expected to be buried in the active site of native E166Cf but largely exposed to the aqueous environment when attached to a peptide fragment resulting from proteolytic digestion. This method was used instead of chemical denaturation because chemical denaturants (e.g., guanidine hydrochloride) can quench the fluorescence of fluorescein (data not shown).

The ability of the proteolytic enzyme trypsin to digest E166Cf was first investigated by CD spectropolarimetry. Figure 6a shows the far-UV CD spectra of E166Cf before and after trypsin digestion. In the absence of trypsin, E166Cf shows negative peaks at 210 and 222 nm, which are characteristic of the α -helix. After trypsin digestion, these peaks vanish, and a new negative peak in the wavelength range of 190–200 nm appears, indicating that the native structure of E166Cf is lost. The proteolytic digestion of E166Cf by trypsin was then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). MALDI-TOF-MS is, in general,

more preferable than ESI-MS in the analysis of proteolytic peptide fragments because time-consuming HPLC separation is not required prior to mass spectrometric measurements. Nine detectable peptide fragments of E166Cf resulting from trypsin digestion were observed (Table S1). A similar profile of peptide fragments was also obtained with the unlabeled E166C mutant under similar experimental conditions (Table S1). The F165-R178 fragment, corresponding to part of the Ω -loop, was obtained after proteolytic digestion of E166C and E166Cf. The mass difference between the F165-R178 fragment derived from E166C and that from E166Cf is 445.1 Da, which is consistent with the molecular mass of the fluorescein label plus a water molecule ($427 + 18 = 445$ Da), as also observed in the previous ESI-MS measurements of E166C and E166Cf. In this context, we noted that formation of protein–water adduct complexes under MALDI-TOF-MS conditions is also a commonly observed phenomenon.³⁶ The MALDI mass spectral data reveal that the fluorescein label is attached to the partial fragment of the Ω -loop, most likely at the Cys166 position, as revealed by the SDS-PAGE assay performed in our previous study.⁵ Taking the mass spectral and CD data together, the fluorescein-labeled Ω -loop is likely to exist as an unfolded peptide fragment in solution after native E166Cf is digested with trypsin.

The fluorescence response of the fluorescein label of E166Cf in the presence of trypsin was then studied. As shown in Figure 6b, the fluorescence signal of E166Cf increases upon trypsin digestion, a proteolytic process that can release the fluorescein label from the confined active site of E166Cf to the external aqueous environment. This observation indicates that the fluorescein label is likely to gain lower water exposure in the active site with respect to the external aqueous environment.

3.5. Mathematical Simulation. In order to estimate the relative contributions of the ES and ES* states to the enhanced fluorescence of E166Cf, mathematical simulations of the concentration/time profiles of different species in the hydrolysis of cefuroxime were performed, using the kinetic parameters determined in the mass spectrometric studies. As shown in Figure S9, the concentration of ES* increases with concomitant decreases in the concentrations of E and ES over the time course. The concentration-time profiles of ES and ES* indicate that the concentration of ES* is, in general, larger than that of ES over the time course; the ratio of $[ES^*]/[ES]$ increases from 2 to 1700 over the time interval of 1–300 s.

3.6. Thermal Denaturation. It has been shown that the replacement of Glu166 in the class A TEM-1 E166N mutant destabilizes the protein structure, rendering the Ω -loop more flexible.²⁷ To investigate whether this phenomenon is also associated with E166Cf, the stabilities of the wild-type enzyme, E166C and E166Cf against thermal denaturation were studied by far-UV CD spectropolarimetry. The midpoints of thermal denaturation (T_m) for the wild-type enzyme, E166C and E166Cf were found to be $56(\pm 1)$, $50(\pm 1)$, and $49(\pm 1)$ °C, respectively. These results indicate that the E166C mutation causes a destabilizing effect on the protein structure and increases the flexibility of the Ω -loop.

4. Discussion

Cysteine is a reactive amino acid that is often strategically incorporated into the structures of proteins for fluorophore

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labeling in the studies of protein–protein^{37–40} and protein–ligand^{6,8,9,12–14,16} interactions. The class A PenPC β -lactamase contains no cysteine^{41,42} and therefore introducing this residue into the structure of this enzyme allows site-specific fluorophore labeling. In this study, the catalytically important Glu166 residue of this wild-type enzyme^{28,30} was replaced with a cysteine and subsequently labeled with the environment-sensitive fluorophore fluorescein-5-maleimide. Recent studies have shown that Glu166 is likely to play critical roles in both the acylation^{27,43–46} and deacylation^{27,30,31,47,48} steps in β -lactam hydrolysis; in the former step, Glu166 is likely to deprotonate the hydroxyl group of Ser70 via the bridging water molecule for β -lactam acylation,^{27,43–46} whereas in the latter step Glu166 appears to act as the general base for hydrolyzing the covalent acyl enzyme–substrate complex.^{27,30,31,47,48} Replacement of this catalytically important residue should, therefore, significantly reduce the activity of the enzyme, thus allowing the fluorophore-labeled E166Cf mutant to serve as a ligand-binding protein (with very low k_{cat}) for biosensing purposes. Moreover, Glu166 is located in the flexible Ω -loop (close to the active site) with its side chain pointing toward the active site.^{17–20} These structural properties allow the fluorophore attached to this residual position to probe local environmental changes within the active site and can reduce the possible steric effect of the fluorophore on the enzyme–substrate binding.

The comparative fluorescence studies of E166Cf and E166C showed that the fluorescein label can sense the local environmental change in the active site induced by antibiotic binding (Figure S8). Moreover, the mass spectrometric and stopped-flow fluorescence experiments revealed that the fluorescence enhancement of E166Cf is very likely to arise from the formation of enzyme–substrate complex (Figure 4e–g). This observation is common to both penicillins (e.g., penicillin G and 6-APA) and cephalosporins (e.g., cefuroxime). With β -lactamase-sensitive antibiotics such as penicillin G, the ES* state of E166Cf is reactive and returns to the E state readily with the generation of acid product, thus leading to the reduction in the ES* population during the course of β -lactam hydrolysis. This phenomenon appears to lead to the restoration of the weak fluorescence of E166Cf, as revealed by the observation that both the mass spectrometric and fluorescence signals decline consistently in the later stage of β -lactam hydrolysis (Figure 4g). In the case of 6-APA and cefuroxime, E166Cf does not show

this phenomenon, an observation consistent with the fact that these antibiotics are resistant to the hydrolytic action of the mutant enzyme (low k_{cat}).

The mechanism by which the binding of antibiotics triggers the fluorescence of E166Cf was unraveled by the molecular modeling studies of the E, ES, and ES* states. An important finding is that the fluorescein label is likely to share a common space with the bicyclic structure of β -lactam antibiotics within the active site (Figure 5a). This observation implies that the fluorescein label has to depart from the active site in order to make room for an incoming antibiotic. Indeed, the protein models of the ES and ES* states reveal that the fluorescein label stays out of the active site in order to avoid the spatial clash with the bound substrate (Figures 1b and 5b). This steric effect is further supported by the finding of the fluorescence study of E166Cf with 6-APA; the binding of 6-APA, which consists of the bicyclic structure only, can also enhance the fluorescence of E166Cf, as similar to the case of penicillin G and cefuroxime (Figure 3d). Taken together, the fluorescein label in the E state appears to occupy, at least in part, the space within the active site where the bicyclic structure of β -lactam antibiotic resides, and this spatial clash is likely to be the major factor inducing the departure of the fluorescein label from the active site upon antibiotic binding.

The substrate-induced movement of the fluorescein label from the active site to the external aqueous environment appears to increase its water exposure, as revealed by the larger solvent-accessible areas of the ES and ES* states compared to that of the E state (Figures 1 and 5). This observation is consistent with the findings of the proteolytic studies of E166Cf analyzed by fluorescence spectroscopy, CD spectropolarimetry, and MALDI-MS; the fluorescein label, which fluoresces more strongly in a more polar environment,^{34,35} shows stronger fluorescence upon digesting native E166Cf into unfolded peptide fragments by trypsin (Figure 6 and Table S1).

The facts that E166Cf gives stronger fluorescence in the ES* state and the fluorescein label gains a similar increase in solvent accessibility in the ES and ES* states imply that, in addition to the covalent ES* state, the noncovalent ES state is also likely to enhance the fluorescence of E166Cf. When E166Cf binds to a β -lactam antibiotic to form the ES complex, the fluorescein label will move away from the active site to avoid the spatial clash with the bound substrate (Figures 1a and 5b). This noncovalent state will then undergo acylation and proceed to the ES* state, where the fluorescein label remains out of the active site (Figure 1b). As a result of gaining higher water exposure, the ES and ES* states give stronger fluorescence with respect to the E state. Upon returning to the E state, the fluorescein label on the Ω -loop enters the vacant active site again. This motion might be hindered due to the bulky nature of the fluorescein label, thus leading to the slow return of the fluorophore. In the E state, the fluorescein label experiences lower water exposure in the active site, thereby restoring its weak fluorescence.

Despite the bulky nature of the fluorescein label, the ability of E166Cf to bind to β -lactam antibiotics is not significantly impaired, as revealed by the similar K_d values of E166C and E166Cf with 6-APA and cefuroxime (Table 1). This surprising observation is presumably due to the flexible nature of the Ω -loop.^{17–20} In this regard, it is interesting to note that replacement of Glu166 in the Ω -loop of the class A TEM-1 β -lactamase with an asparagine can increase the mobility of the Ω -loop and hence destabilize the protein structure.²⁷

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Previous structural studies of the class A RTEM-1 and TEM-1 β -lactamases have shown that the side-chain carboxylic group of Glu166 forms hydrogen bonds with the side-chain nitrogen atoms of Lys73 and Asn170,^{31,43,49} which are highly conserved residues in many class A β -lactamases, including the PenPC β -lactamase used in the present study.^{41,42} Moreover, in the E166N mutant of the TEM-1 β -lactamase, the replacement of Glu166 in the Ω -loop is likely to weaken the hydrogen-bonding interactions, thus increasing the flexibility of the Ω -loop and destabilizing the structure of the enzyme; the T_m values of the wild-type form and E166N mutant of TEM-1 were found to be 51.1($\pm$ 0.2) and 43($\pm$ 1.4) $^{\circ}$ C, respectively.²⁷ These perturbing structural effects might also occur in the E166C mutant of the PenPC β -lactamase because the thermal stabilities of the Glu166-substituted mutants of the PenPC and TEM-1²⁷ β -lactamases are both reduced (compared to their respective wild-type structure) as a result of the replacement of Glu166. In the case of E166Cf, the E166C mutation and the fluorophore conjugation appear to cause a similar destabilizing effect on the protein structure, as revealed by the similar decrease in thermal stability of E166Cf compared to that of the wild-type enzyme. This structural perturbation is likely to increase the flexibility of the Ω -loop and hence compensate for the possible steric effect of the bulky fluorescein label on the enzyme–substrate binding. Analogous to this case, some β -lactamase variants in the class A TEM family have been found to be able to improve the binding interactions with bulky cephalosporins by relaxing the conformation of their Ω -loop through the replacement of the Ω -loop residue (e.g., Arg164) responsible for forming salt bridges with the rest of the protein structure.^{50–52} With the increased flexibility of the Ω -loop, E166Cf appears to be able to largely restore its substrate-binding ability even

though its active site is considerably shielded by the fluorophore in the free enzyme state.

5. Conclusions

In this study, we have studied the biosensing mechanism of the nonallosteric E166Cf mutant toward β -lactam antibiotics. The catalytic Glu166 residue was replaced with a cysteine, which was subsequently labeled with environment-sensitive fluorescein. This residue is located in the flexible Ω -loop with its side chain pointing toward the active site.^{17–20} The replacement of Glu166 further increases the flexibility of the Ω -loop. With these advantageous structural properties, the attached fluorescein label can stay close to the active site to sense antibiotic binding without significantly impairing the substrate binding ability of the mutant enzyme. In the E state, the fluorescein label is likely to occupy, at least in part, the active-site space where the bicyclic structure of β -lactam antibiotics resides. This spatial clash appears to cause the fluorescein label to depart from the active site when an antibiotic enters this local region. As a result of this subtle motion, the fluorescein label in the ES and ES* states experiences higher water exposure and hence gives stronger fluorescence with respect to the E state. After hydrolyzing the bound substrate, the fluorescein label returns to the vacant active site and restores its weak fluorescence.

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Supporting Information Available: Experimental details for fermentation, molecular modeling of E166Cf, and mass spectral data for E166C and E166Cf. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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