Rational Design of a Novel Fluorescent Biosensor for β-Lactam Antibiotics from a Class A β-Lactamase

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Detection of trace antibiotic contaminants in food (e.g., milk) is important, as the ubiquitous presence of antibiotics can have serious health consequences on the humankind ranging from allergic reactions to the evolution of antibiotic-resistant bacteria.[1,2] β-Lactam antibiotics such as penicillins and cephalosporins, which are widely used in veterinary medicine to treat bacterial infections,[3] are the major contaminants. Conventional screening tests such as the Charm test,[4] the Penzym test,[5] and the BetaScreen test[6] are largely semiquantitative. Analysis of antibiotics by HPLC[7] usually requires tedious sample pretreatments. The fiber optic[8] and electrochemical[9] β-lactam sensors that measure pH changes during hydrolysis of the β-lactam ring by β-lactamases[10] are subject to interference from change in environmental pH. It is thus highly desirable to have a simple, quantitative, and sensitive method that can detect antibiotics at trace levels in a large number of samples.

The rational design of biosensors based on induced conformational changes of proteins is an emerging approach for detecting small ligands and ions.[11−14] The proteins selected for biosensor construction are usually allosteric enzymes capable of ligand binding but without catalysis, and therefore, hydrolytic enzymes are seldom used.[14a] Bacterial class A β-lactamases are extremely powerful enzymes that efficiently bind to and hydrolyze a large variety of β-lactam antibiotics.[10] Neither substrate binding nor catalysis has been reported to induce large conformational changes in these enzymes. Thus, the rational design of biosensors to detect small ligands based on such enzymes remains a great challenge. Although β-lactamases are not classified as allosteric enzymes, recent reports indicate that the Ω-loop, a loosely packed 17-residue segment (residues 163−179) adjacent to the active site of class A β-lactamases, is flexible and that its motion is essential for the enzymatic reaction.[15] The Ω-loop contains an important active-site residue Glu-166, the mutation of which would impair but still allow the enzyme to undergo acylation with β-lactam antibiotics; nevertheless, the deacylation step will become very slow compared to the wild-type enzyme.[16,17] Moreover, crystal structures of the enzyme show that the Glu-166 side chain is pointing toward the active site.[16b] We reasoned that if an environment-sensitive fluorescent probe is placed specifically at the 166 position, an enzyme with drastically suppressed hydrolytic activity that can also function as a biosensor can be constructed.

To demonstrate this concept, a mutant of β-lactamase I from *Bacillus cereus* 569/H, E166C, was first constructed by site-directed mutagenesis in which the Glu-166 residue on the Ω-loop responsible for activating the water molecule in the hydrolysis of the β-lactam ring was replaced with a cysteine residue.[17] The catalytic efficiency

Scheme 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$
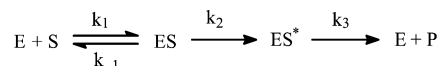
of this mutant for different β-lactams has been shown to be over 1800-fold lower than those of the wild-type enzyme.[17] Thus, the enzyme−substrate complex is sufficiently stable to allow the mutant to function as a biosensor. The E166C mutant was then labeled with the thiol-specific fluorophore fluorescein-5-maleimide to give the labeled mutant E166Cf. As the wild-type β-lactamase I contains no cysteine residue, this single-point mutation allows the attachment of thiol-reactive fluorophore to the specific site on the enzyme. The catalytic efficiencies k_{cat}/K_m (where k_{cat} is the turnover number and K_m is the Michaelis−Menten constant) of E166Cf toward various β-lactam antibiotics are comparable to those of the E166C mutant, indicating that the active-site structure is conserved after labeling with fluorescein.

E166Cf is only weakly fluorescent in aqueous buffer. When β-lactam antibiotics are added to a 0.05 M phosphate buffer solution (pH 7.0) of the labeled mutant (0.12 μM), the fluorescence intensity at 515 nm increases as a function of the antibiotic concentration (Figures 1A and 1B). The increase in fluorescence intensity can be observed even when the antibiotic concentration is as low as 0.05 μM, indicating that the biosensor is extremely sensitive in detecting antibiotics at trace levels. The time-resolved fluorescence changes at 515 nm under different concentrations of penicillin G and cefuroxime are shown in Figures 1C and 1D, respectively. For penicillin G, the fluorescence intensity first increases upon the addition of antibiotic, levels off to a plateau, and then slowly declines. This pattern is observed for all the antibiotics under the penicillin family being tested. For cephalosporins such as cefuroxime, time-resolved fluorescence measurements indicated that the fluorescence signal takes a longer time to reach the maximum intensity, but no subsequent decline in fluorescence intensity was observed even after 1 h. The fluorescence signal changes from the biosensor can be rationalized by the enzymatic reaction shown in Scheme 1, where E is the enzyme, S is an antibiotic substrate, ES is the noncovalent enzyme−substrate complex, ES* is the acyl− enzyme adduct, and P is the hydrolyzed product. If the increase in fluorescence signal upon the addition of antibiotics is attributed to a change in the local environment of the fluorescein molecule caused by the formation of the ES and ES* complexes, the subsequent hydrolysis of penicillin and release of hydrolyzed product will restore the original weak fluorescence signal. The time-resolved fluorescence changes for cephalosporins are consistent with the fact that cephalosporins are poorer substrates than penicillins for the class A β-lactamases (with much smaller k_1 and k_3).[10,17]

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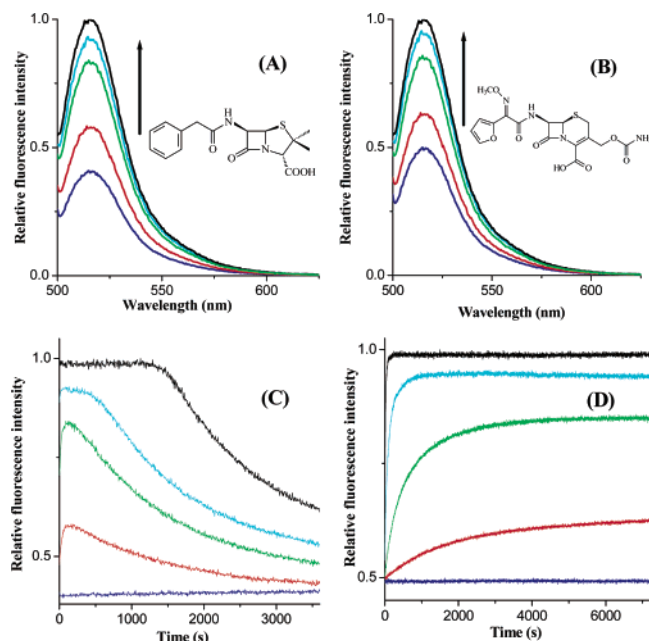
Figure 1. Fluorescence spectra of E166Cf in the presence of penicillin G (A) and cefuroxime (B) in 50 mM phosphate buffer (pH 7.0). The corresponding time-resolved fluorescence changes in the presence of penicillin G and cefuroxime as monitored at 515 nm are shown in panels C and D, respectively. Navy: 0.12 μM E166Cf enzyme. Red, green, light blue, and black, after addition of 0.1, 1.0, 10, 100 μM antibiotics respectively.
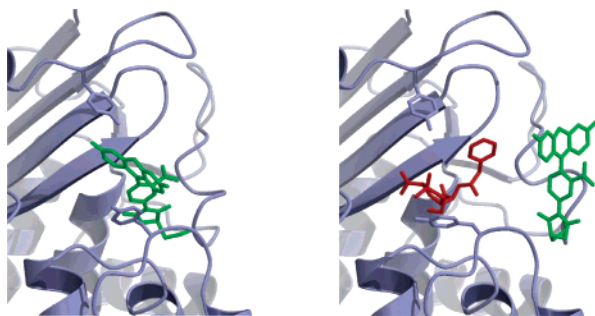
Figure 2. Molecular models of the fluorescein label (green) on E166Cf before (left) and after (right) binding with penicillin G (red).

To understand the mechanism of the increase in fluorescence signal upon binding of antibiotics, a molecular model was constructed for the penicillin G substrate. As shown in Figure 2, the fluorescein molecule lies inside the pocket close to the binding site of the E166Cf mutant. Upon binding of penicillin G, the flexibility of the Ω-loop allows the fluorescein to move outside and be more exposed to solvent molecules. On the basis of the molecular model, the solvent-accessible surface area of residue 166 was calculated to increase by 170 Å^2 after binding with penicillin G. This is consistent with previous reports that the fluorescence intensity of fluorescein increases upon exposure to a more polar environment.[18]

Preliminary experiments indicate that the E166Cf mutant can detect β-lactam antibiotics as low as 0.1 μM in centrifuged milk samples. As expected, this biosensor also works for detecting β-lactamase inhibitors such as clavulanic acid and sulbactam, and hence may find its application in the pharmaceutical industry for the discovery of new inhibitors. By doing this in a 96-well microplate, a high-throughput screening of inhibitor candidates can be achieved.

In conclusion, a sensitive and reagentless biosensor for β-lactam antibiotics has been constructed from a modified class A β-lactamase. This biosensor provides a more quantitative assay of β-lactams than most conventional colorimetric tests, yet is simpler in operation than the HPLC method. We have demonstrated that turn-on fluorescent biosensors for direct detection of small ligands can be constructed by placing environment-sensitive fluorophores on flexible loops of enzyme mutants with drastically impaired activity. This rational design strategy opens a possibility for converting highly active and nonallosteric enzymes into substrate-binding proteins for biosensing purposes.

Supporting Information Available: Experimental details on the preparation of E166Cf and molecular modeling; fluorescence spectroscopic data for different antibiotics and inhibitors and in centrifuged milk sample; k_{cat} and K_m values; and mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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