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A FLUORESCENT CARBAPENEM FOR STRUCTURE FUNCTION STUDIES OF PENICILLIN-BINDING PROTEINS, B-LACTAMASES AND B-LACTAM SENSORS

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Abstract

By reacting fluorescein isothiocyanate with meropenem, we have prepared a carbapenem-based fluorescent β -lactam. Fluorescein-meropenem binds both penicillin binding proteins and β -lactam sensors, and undergoes a typical acylation reaction in the active site of these proteins. The probe binds the class D carbapenemase OXA-24/40 with close to the same affinity as meropenem, and undergoes a complete catalytic hydrolysis reaction. The visible light excitation and strong emission of fluorescein render this molecule a useful structure-function probe through its application in SDS-polyacrylamide gel electrophoresis assays, as well as solution-based kinetic anisotropy assays. Its classification as a carbapenem β -lactam and the position of its fluorescent modification render it a useful complement to other fluorescent β -lactams, most notably Bocillin FL. In this study we show the utility of fluorescein-meropenem by using it to detect mutants of OXA-24/40 that arrest at the acyl-intermediate state with carbapenem substrates, but maintain catalytic competency with penicillin substrates.

Keywords

β -lactamase; penicillin-binding protein; fluorescent-modification; carbapenem

Introduction

The study of bacterial penicillin-binding proteins (also known as PBPs, or transpeptidases) has been greatly enhanced by the creation of fluorescently-modified β -lactam ligands for use

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as structure-function probes. Most notable among these reagents is the fluorescent penicillin, Bocillin FL, created by the conjugation of a bodipy fluorophore to a standard penicillin [1]. Bocillin FL has been used to define the PBP profiles of intact bacterial membranes [1; 2] and to measure the affinity of unlabeled β -lactams to PBPs via competition assays [3; 4]. It has also been used to determine the acylation and deacylation rate constants for catalytically impaired β -lactamases [3; 5]. The use of Bocillin FL with β -lactamases, however, is limited by its very identity as a penicillin as it is rapidly hydrolyzed by those enzymes. Moreover, as with all ligands modified by fluorescent probes, the attachment of the bodipy moiety on the penicillin side-chain may impact the results through supplementary interactions with the active site. To expand the utility of these types of probes, we sought to modify the carbapenem meropenem (Fig. 1A) with the amine-modifying reagent fluorescein isothiocyanate (FITC). Meropenem possesses an amine on the pyrrolidine ring of its side-chain, and isothiocyanates react readily with amines to yield a stable thiourea linkage (Fig. 1B). The FITC modification of meropenem would be expected to be on the opposite side of the β -lactam compared to the position of the bodipy attachment point in Bocillin FL. In this study we show that fluorescein-meropenem is useful as a structure-function probe for a wide variety of penicillin-binding proteins and β -lactamases.

Materials and Methods

Synthesis of fluorescein-meropenem

Fluorescein-meropenem (FM) was prepared by mixing 44 mg of fluorescein-isothiocyanate (FITC isomer I, Invitrogen) with 45 mg meropenem/14 mg sodium bicarbonate (Merrem, Astrazeneca) in 1.0 mL N,N-dimethylformamide (DMF, Sigma). The reaction proceeded at room temperature overnight with stirring in the dark. The product was isolated using preparative thin-layer chromatography on Silica Gel (Analtech Uniplat) using 85% ethyl acetate:15% acetic acid as a mobile phase. Under these conditions, the product had an R_f of ~ 0.6 , FITC ran at the solvent front, and both meropenem and hydrolyzed fluorescein-meropenem remained at the origin. Silica gel was scraped off the plate and the product was eluted with ~ 3 ml of DMF. The addition of excess ethyl acetate (9-fold v/v to DMF) caused precipitation of the product, which was then collected by centrifugation. Pellets of purified fluorescein-meropenem were stored at -80°C and were found to possess unchanged response in all biochemical assays (see below) for at least two months. Prior to use, the product was resuspended in 50 mM NaH_2PO_4 pH 7.4. The concentration of purified FM was determined using the extinction coefficient of fluorescein at 496 nm ($68,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$) in 50 mM NaH_2PO_4 , pH 8.0 [6].

^1H NMR spectra

The structure of the FITC-labeled meropenem product obtained after reaction and purification was determined by standard one-dimensional NMR analysis. The ^1H NMR spectra were obtained on a Jeol Eclipse 300 MHz FTNMR spectrometer at 25°C . The purified labeled meropenem was dissolved in D_2O (Sigma-Aldrich) in a 5 mm NMR tube. The total volume of sample was 0.7 ml, and had a concentration of approximately 1 mg/ml. Sixteen scans were collected with standard pulse sequences and the data processed using Delta software. Successful reaction and purification (labeled samples were typically 90–95%

pure by NMR) was verified by the presence of select FITC and meropenem proton signals (see Supplemental Material) [7; 8; 9; 10].

Protein expression constructs

Expression vectors have been described previously for OXA-1 [11] and OXA-24 [12]. A similar expression system for AmpC was created using the Polymerase Chain Reaction (PCR) to amplify the coding region for residues (20–361) of AmpC [13] (representing the mature, proteolyzed form). The PCR primers were designed such that NdeI and BamHI restriction sites were introduced at the 5' and 3' termini, and the resulting fragment was ligated into the same sites of pET-24a. The gene for TEM-1 (from the pUC19 plasmid) was also used as a template for PCR. BamHI and HindIII restriction sites were introduced at the 5' and 3' ends of the mature form of TEM-1 (residues 24–290), and the gene was ligated into the same sites of pET-28a. The soluble penicillin-binding domain of the BlaR1 protein (BlaR1^S) of *Staphylococcus aureus* (the kind gift of Dr. Shahriar Mobashery, University of Notre Dame) [14] was excised from the pET-24a vector using NdeI and XhoI and ligated into the same sites of pET-28a. The soluble periplasmic penicillin-binding domain of PBP3 from *Acinetobacter* sp. strain ATCC 27244 (PBP3^S; residues 64–609) was previously inserted into pET28a [4; 15]. The pET-28a constructs were prepared to allow for the production of a 6X-histidine fusion protein and subsequent purification by metal-chelate affinity chromatography. All constructs were introduced into BL21(DE3) *Escherichia coli* (*E.coli*) cells for expression.

Protein purification

Expression and purification of OXA-1, OXA-24/40 and AmpC were all carried out using carboxymethyl cellulose cation-exchange chromatography as described previously for the purification of OXA-24/40 [12]. Growth, induction and lysis of BL21(DE3) *E.coli* cells containing the pET-28a constructs of 6X-His-TEM-1, 6X-His-AmpC and 6X-His-BlaR1^S were also identical to the procedure in [12], and the purification was as follows. A 1×3 cm His-Pur Cobalt column (ThermoScientific) was equilibrated with 20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl pH 7.4. Clarified lysates were applied to the column and washed with ~ 20 ml 20 mM Tris-HCl, 25 mM imidazole, 500 mM NaCl pH 7.4. Pure 6X-His-tagged protein was eluted with 20 mM Tris-HCl, 200 mM imidazole, 500 mM NaCl pH 7.4 and dialyzed overnight at 4°C in 50 mM NaH₂PO₄ pH 7.0. Purified protein (95% by SDS-PAGE) was snap frozen in liquid nitrogen and stored at –80°C until needed. The concentration of all purified proteins was determined by measuring the absorbance at 280 nm and using an extinction coefficient calculated by the method of Gill and von Hippel [16].

SDS-polyacrylamide gel electrophoresis assays for detection of acyl-intermediates

Acylenzyme intermediates formed between FM and various proteins were detected using denaturing gel electrophoresis. Samples of 1 μM purified protein were incubated with 20 μM FM in 50 mM NaH₂PO₄ pH 7.0 for 3 minutes. Competitive blocking was achieved by preincubation of 2.8 mM unlabeled meropenem for 3 minutes before the addition of FM. For all labeling reactions, SDS sample buffer was added to each tube and the samples were separated by 10% SDS-PAGE. Fluorescence emission was captured using a DR46B

Transilluminator (maximum emission 460 nm; Clare Chemical Research) with an amber screen and a SYBR green (515–570 nm) emission filter. Gels were subsequently stained with Coomassie Brilliant Blue G. A similar method was used for the detection of acyl intermediates between OXA-24 V130 and L168 variants and Bocillin FL or fluorescein-meropenem. Aliquots of 2 μ g of each purified protein were incubated with 6.9 μ M Bocillin FL or 6.9 μ M FM in 50 mM NaH₂PO₄ pH 7.0 and 25 mM NaHCO₃ for 10 minutes, followed by the addition of an excess of doripenem (2.3 mM) for an additional 10 minutes.

Anisotropy Assays

Anisotropy was monitored using a Photon Technology Incorporated QuantaMaster 7 fluorimeter in T-format. The excitation monochromator was set to 496 nm (slit-width 3 nm) and the emission monochromator was 515 nm (slit-width 8 nm). Fluorescein-meropenem (50 nM) was added to 0.5 ml of 50 mM NaH₂PO₄ pH 7.4 in a semi-micro fluorescence cuvette (2 $\times$ 10 mm), and the anisotropy was measured over time. Aliquots of BlaR1, PBPS, AmpC or OXA-24/40 were added and the anisotropy continuously monitored. To show that the anisotropy change was due to FM binding in the active site, an excess amount of unlabeled carbapenem (50 μ M doripenem) was added prior to the addition of protein. The decay of anisotropy observed after the addition of OXA-24/40 was fit to a single exponential function to determine a deacylation time constant. The anisotropy change measured upon addition of AmpC was fit to the sum of two exponential functions.

Kinetic assays

Steady state kinetic analysis for OXA-24/40 with meropenem as a substrate was performed as previously described [17]. The affinity constant (K_S) for fluorescein-meropenem binding to OXA-24/40 was measured using FM as a competitive inhibitor of ampicillin hydrolysis, as previously described [17; 18].

Results and Discussion

In order for this novel probe to have broad utility, it would ideally bind to a wide variety of proteins known to interact specifically with β -lactams. We incubated FM with purified preparations of a penicillin-binding protein (the soluble domain of PBP3 from *Acinetobacter* sp. strain 27244), a β -lactam sensor protein (the soluble domain of BlaR1 from *Staphylococcus aureus*) and three β -lactamase proteins: TEM-1 (class A), AmpC (class C) and OXA-1 (class D). Covalently-modified proteins were separated from free intact FM using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using a transilluminator. The results (Fig. 2) show that all of these proteins were strongly acylated by FM in less than the 3 minute duration of the reaction. Inclusion of a large excess of unlabeled meropenem as a competitor blocked the labeling event for all proteins, indicating that FM binds in the active site.

We then went on to show that we could directly monitor the interaction of the fluorescently-modified meropenem with many of these proteins using fluorescence anisotropy. As seen in Fig. 3a, the anisotropy of free FM is very low when in buffer solution ($\sim$ 0.025). The addition of BlaR1^S or PBP3^S resulted in a rapid and irreversible rise in anisotropy that likely

represents the non-covalent association and/or covalent acylation of the ligand and protein (Fig. 3a and 3b). As with the gel assay, the addition of excess unlabeled carbapenem completely eliminated the signal change. Interestingly, we also observed a rise in anisotropy when FM was challenged with β -lactamases, but the signal change was reversed over time (Fig. 4). In the case of AmpC (Fig. 4a), the time-course of anisotropy decline was very slow, as one would expect for an enzyme known to have very low catalytic efficiency against carbapenems [19]. The anisotropy change over time could be fit to a double exponential function showing that this technique will likely be useful for measuring acylation and deacylation rates of β -lactamases much as Shapiro *et al.* did for a catalytically-compromised variant of OXA-10 using Bocillin FL [3]. For the known carbapenemase OXA-24/40 however, the increase and subsequent decline in anisotropy was almost too fast to observe without resorting to fast mixing schemes (Fig. 4b; upper panel). The anisotropy decline that could be observed fit well to a single exponential function, with a rate constant ($0.042 \pm 0.002 \text{ s}^{-1}$) (Fig. 4b; lower panel) that was only slightly slower than the overall catalytic rate of this enzyme with unlabeled meropenem ($0.13 \pm 0.01 \text{ s}^{-1}$). We also compared the binding affinity of FM and meropenem to OXA-24 using a kinetic competition method with ampicillin as a reporter substrate [17]. The K_S value for FM ($43 \pm 5 \text{ nM}$) was only slightly higher than that of unmodified meropenem ($18 \pm 3 \text{ nM}$). Thus the attachment of a bulky fluorescein moiety to the side-chain of meropenem has very little effect on the affinity or the rate-limiting deacylation step for that substrate with OXA-24/40. This is quite surprising as the OXA-24/40 enzyme is known to have a highly constricted active site due to the presence of a hydrophobic bridge formed by residues Tyr112 and Met223 [12; 20]. This high-affinity binding to OXA-24/40 and its ability to bind across multiple classes of β -lactamases and PBPs suggest that this reagent may be useful as a reporter in high throughput screening assays designed to identify novel β -lactamase and transpeptidase inhibitors.

The carbapenem core of FM suggests another potential use. Much effort has been directed toward understanding the mechanism of inhibition of narrow-spectrum β -lactamases by carbapenems, and the mode by which carbapenemases are able to effectively hydrolyze these clinically important antibiotics. A reagent based on a carbapenem scaffold may be able to illuminate those mechanisms in a way that a penicillin-based probe cannot. For instance, we recently produced a large number of amino acid substitutions at the Val130 and Leu168 positions of OXA-24/40, two sites that have been suggested in multiple studies to play a role in the ability of that enzyme to deacylate carbapenems [21; 22; 23]. We used the SDS-PAGE acyl-enzyme detection assay to study how these substitutions affect the interaction of FM with OXA-24/40. We ran these assays in the presence of 25 mM bicarbonate, as some of these substitutions have been shown to decrease the N-carboxylation of Lys84, a modification known to be important in that residue's role as a general base. We also carried out these assays in single-turnover conditions (*ie.* addition of unlabeled carbapenem after Bocillin FL or fluorescein-meropenem binding). Together, these conditions ensure that only those mutant enzymes that display greatly attenuated deacylation rates will be observed as fluorescent acyl-intermediates.

As expected, the use of Bocillin FL with wild-type OXA-24/40 produced no acyl-intermediate, while a known deacylation-deficient variant of the same enzyme (Lys84Asp)

showed very strong labeling [12]. All other Val130 mutants showed no fluorescent labeling, suggesting maintenance of deacylation activity. An identical experiment carried out with FM showed very different results. All Val130 substitutions showed some degree of fluorescence labeling, but the Val130Asp, Val130Thr and Val130Asn substitutions most severely reduced deacylation. Very similar results were observed when both Bocillin FL and fluoresceinmeropenem were used with a panel of Leu168 substitutions. Again, Bocillin FL deacylation was not affected, while all Leu168 mutations led to some degree of FM deacylation-deficiency. In this case, the two substitutions that most closely match the branched chain aliphatic wild-type leucine residue (Leu168Ile and Leu168Val) showed the least signal. This is consistent with these two mutations having deacylation activity higher than the other mutants.

Critically, both Leu168 and Val130 come in close contact with the hydroxyethyl moiety present in all carbapenems. The position of fluorescein modification of meropenem at the other end of the molecule, is fortuitously far from the area of this crucial interaction. The high sensitivity of carbapenem deacylation to substitutions at both positions is consistent with previous suggestions that subtle interactions between these residues and the hydroxyethyl moiety of the drug play an important role in activating deacylation [12; 22; 23].

In summary, the novel fluorescent carbapenem fluorescein-meropenem shows excellent binding properties across a wide-variety of proteins known to interact with β -lactam substrates. Its carbapenem structure and position of fluorescent labeling render it a useful complement to other reagents used for labeling of PBPs and β -lactamases, most notably, Bocillin FL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of (A) meropenem and (B) fluorescein-meropenem, formed from the reaction of meropenem and fluorescein isothiocyanate (isomer I).

Figure 2.

Detection of fluorescein-meropenem acyl-intermediates with the soluble domain of PBP3 from *Acinetobacter* sp. strain ATCC 27244 (PBP3^S), the soluble domain of the β -lactam sensor BlaR1 from *Staphylococcus aureus* (BlaR1^S) and the β -lactamases TEM-1, AmpC and OXA-1. After incubation of 20 μ M fluorescein-meropenem with 1.0 μ M pure protein for three minutes, samples were separated from free FM by SDS-PAGE. Unlabeled meropenem (2.8 mM) was added as a competitive block to the lanes marked +. The top panel represents fluorescence emission and the bottom panel shows staining with Coomassie Blue.

Figure 3.

The use of anisotropy to monitor the interaction of fluorescein-meropenem with PBP3^S (A) and BlaR1^S β -lactam sensor (B). Anisotropy was monitored for 50 nM fluorescein-meropenem alone (at time 0) and after the addition of 200 nM protein (indicated by arrow). Solid points and unfilled markers represent the absence and presence respectively of excess (50 μ M) doripenem used as a competitive block.

Figure 4.

The use of anisotropy to monitor the interaction of fluorescein-meropenem with two different β -lactamases. (A) The anisotropy of a 50 nM solution of fluorescein-meropenem was monitored and at the time indicated by the arrow, samples of the non-carbapenemase β -lactamase AmpC were added (red, 2 μ M; green, 1 μ M; blue, 0.5 μ M). Each of these data sets was fit to a double exponential function (solid line). (B) The experiment was repeated with the carbapenemase OXA-24/40. Solid points and unfilled markers represent the absence and

presence respectively of excess (50 μM) doripenem used as a competitive block. The rapid decay of the anisotropy was fit to a single exponential function (solid line).

Figure 5.

Comparison of penicillin and carbapenem deacylation-deficiency for OXA-24/40 V130 and L168 mutants. Pure preparations of OXA-24/40 mutants (substituted as indicated at position V130 or L168) were incubated with FM or Bocillin FL for 3 minutes followed by the addition of excess doripenem and a further incubation of 10 minutes. The reaction was stopped by the addition of sample buffer, and the samples were separated by 10% SDS-PAGE.