Identification of Residues in \( \beta \)-Lactamase Critical for Binding \( \beta \)-Lactamase Inhibitory Protein*

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\( \beta \)-Lactamase inhibitory protein (BLIP) is a potent inhibitor of several \( \beta \)-lactamases including TEM-1 \( \beta \)-lactamase (\( K_i \) = 0.1 nM). The co-crystal structure of TEM-1 \( \beta \)-lactamase and BLIP has been solved, revealing the contact residues involved in the interface between the enzyme and inhibitor. To determine which residues in TEM-1 \( \beta \)-lactamase are critical for binding BLIP, the method of monovalent phase display was employed. Random mutants of TEM-1 \( \beta \)-lactamase in the 99–114 loop-helix and 235–240 B3 \( \beta \)-strand regions were displayed as fusion proteins on the surface of the M13 bacteriophage. Functional mutants were selected based on the ability to bind BLIP. After three rounds of enrichment, the sequences of a collection of functional \( \beta \)-lactamase mutants revealed a consensus sequence for the binding of BLIP. Seven loop-helix residues including Asp-101, Leu-102, Val-103, Ser-106, Pro-107, Thr-109, and His-112 and three B3 \( \beta \)-strand residues including Ser-235, Gly-236, and Gly-238 were found to be critical for tight binding of BLIP. In addition, the selected \( \beta \)-lactamase mutants A113L/T114R and E240K were found to increase binding of BLIP by over 6- and 11-fold, respectively. Combining these substitutions resulted in 550-fold tighter binding between the enzyme and BLIP with a \( K_i \) of 0.40 pm. These results reveal that the binding between TEM-1 \( \beta \)-lactamase and BLIP can be improved and that there are a large number of sequences consistent with tight binding between BLIP and \( \beta \)-lactamase.

Since the introduction of \( \beta \)-lactam antibiotics, bacterial resistance to these agents has become an increasing problem (1, 2). The production of the enzyme \( \beta \)-lactamases by both Gram-positive and Gram-negative bacteria is the most common mechanism of resistance to \( \beta \)-lactam antibiotics (3). \( \beta \)-Lactamases hydrolyze the amide bond of the \( \beta \)-lactam antibiotic to create an ineffective antimicrobial agent (3). \( \beta \)-Lactamases sometimes have multiple \( \beta \)-lactamase domains (4, 5). Genes encoding these enzymes have been found on plasmids, transposons, and bacterial chromosomes (4, 6). The most prevalent plasmid-mediated \( \beta \)-lactamase in Gram-negative bacteria is the TEM-1 \( \beta \)-lactamase from class A (7). Like other class A \( \beta \)-lactamases, TEM-1 \( \beta \)-lactamase is capable of hydrolyzing both penicillins and cephalosporins (2, 8, 9). To overcome the drug resistance mediated by TEM-1 \( \beta \)-lactamase, extended-spectrum antibiotics, including aztreonam, cefotaxime, and ceftazidime were developed. However, soon after their introduction, selective pressure resulted in the emergence of variant \( \beta \)-lactamases able to hydrolyze these antibiotics (10). Examination of these enzymes revealed that amino acid substitutions such as R164S, G238S, and E240K in TEM-1 \( \beta \)-lactamase resulted in altered substrate specificity of the enzyme (11–13). The emergence of these enzymes is an additional threat to antimicrobial therapy since plasmids encoding these \( \beta \)-lactamase variants are easily transferable to unrelated bacteria (1).

An additional strategy that has been employed to combat antimicrobial resistance is the use of \( \beta \)-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (14). Although not capable of antimicrobial activity themselves, these suicide inhibitors are used in conjunction with various \( \beta \)-lactam antibiotics to bind \( \beta \)-lactamase and prevent the hydrolysis of the antibiotic, thereby restoring the therapeutic value to the antimicrobial agent. Unfortunately, \( \beta \)-lactamase variants have been identified that are resistant to these enzyme inhibitors while still retaining the ability to hydrolyze \( \beta \)-lactam antibiotics (15, 16).

The small molecule \( \beta \)-lactamase inhibitor, clavulanic acid, was initially purified from the soil bacterium Streptomyces clavuligerus (17). This organism also produces a protein inhibitor of \( \beta \)-lactamase called the \( \beta \)-lactamase inhibitor protein (BLIP) (18). BLIP is a 165-amino acid protein composed of two tandemly repeated domains (19). It has been shown to be a potent inhibitor of class A \( \beta \)-lactamases from both Gram-positive and Gram-negative bacteria, including TEM-1 \( \beta \)-lactamase (\( K_i \) = 0.1 nM) (20). The co-crystal structure of TEM-1 \( \beta \)-lactamase complexed with BLIP has been solved, revealing the mode of inhibition by BLIP and the contact residues involved in the interface between the enzyme and inhibitor (21). The x-ray structure shows that BLIP binds to a negatively charged loop-helix region composed of residues 99–112 just outside the active site pocket of TEM-1 \( \beta \)-lactamase (Fig. 1). Sequence alignment of \( \beta \)-lactamases that are inhibited by BLIP reveals an overall lack of amino acid sequence conservation in this region (21). The ability of BLIP to bind to such a variety of class A \( \beta \)-lactamases is believed to be due in part to an extensive layer of water molecules that are trapped between the inhibitor and the 99–112 loop-helix region of the enzyme. The relative importance of the 99–112 residues of TEM-1 \( \beta \)-lactamase for binding BLIP is unknown. It has been proposed, however, that the only critical residues in this region for binding with BLIP are the aromatic residue at position 105 and the conserved proline at position 107 (21).

In addition to blocking the enzyme-active site by binding the

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‡The abbreviations used are: BLIP, \( \beta \)-lactamase inhibitor protein; BSA, bovine serum albumin; mal, maltose.
99–112 loop-helix region of TEM-1 β-lactamase, BLIP also inserts two turns, one from each domain, directly into the active site of the enzyme (21). The main contact residues in the active site of TEM-1 β-lactamase involve the B3 β-strand residues 234–240 (Fig. 1). The β-hairpin turn of domain 1 of BLIP is stabilized through several van der Waals contacts made with residues 235–238 of β-lactamase in addition to four strong hydrogen bonds made by Asp-49 of BLIP with residues in the enzyme that are critical for substrate binding and catalysis. The turn of domain 2, in particular Phe-142, also makes several contacts with TEM-1 β-lactamase. Since β-lactamases inhibited by BLIP are highly conserved in the 234–240 region, these residues have been hypothesized to be the crucial determinants for the binding and inhibition by BLIP (21).

In order to circumvent bacterial resistance and thereby continue the effectiveness of antimicrobial therapy, new antibiotics and inhibitors are needed. Understanding the molecular details of TEM-1 β-lactamase and BLIP binding may aid in the development of inhibitors and antibiotics designed to mimic the BLIP–β-lactamase interaction (20). In this study we have developed and used a phage display system to determine the residues in the 99–112 loop-helix and the 234–240 B3 β-strand of TEM-1 β-lactamase that are critical for the tight binding interaction with BLIP (Fig. 1).

**Experimental Procedures**

**Bacterial Strains and Plasmids—**Escherichia coli XL1-Blue (22) [recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, F’ proAB lacZMD15, Tn10 (tet’)] (Stratagene, Inc.) was used for transformation of ligation reactions and to produce the initial, unpanned bacteriophage stocks. E. coli TG1 (23) [F’ traD36’, lacI, Δ(lacZ) M15 proA1 B’ /supE Δ(hsdM’ merB’ ) (r- m- MerB’) thi Δ(lac proAB)] was used for production, amplification, and titering of bacteriophage stocks. E. coli RB791 (24, 25) (strain W3110 lacFL8) was used to express and purify β-lactamase mutants. The β-lactamase random libraries encompassing regions 99–114 and 232–240 were previously constructed in the pBG66 plasmid as described (26, 27). The pBG66 vectors harboring the β-lactamase mutants A237T and the double mutant L113A/T114R were selected from random libraries of β-lactamase as described previously (27, 28). The phagemid vector pG3-SPT was constructed previously by the insertion of a spectinomycin cassette into the cat gene of pG3-CMP as described (29).

**Construction of Phage Display Libraries—**For monovalent phage display of β-lactamase random libraries including regions 99–114 and 232–240, the phagemid vector pG3-CMP was used (29). The β-lactamase random library 103–105, consisting of three consecutive random codons 5’-NNN-NNN-NNN-3’ (N = A, G, C, or T) in the vector pBG66, was constructed as described previously (30). The remaining β-lactamase gene libraries were constructed as described (27), each consisting of three consecutive randomized codons 5’-NNN-NNN-NNN-3’ (S = G or C) in vector pBG66. The β-lactamase gene for each library was polymerase chain reaction-amplified using the following primers: PD-bla1, 5’-CGGGGAGCTCGTTTCTTAGACGTCAGGTGGC-3’; PD-bla2, 5’-CCCCGCTCAGACCACATTGATTACCATGAG-3’. Enzyme restriction sites are underlined; the primer PD-bla1 contains a SacI site, and the primer PD-bla2 contains an XbaI site. The polymerase chain reaction product from each β-lactamase random library was digested with SacI and XbaI, gel-purified, and ligated into the SacI/XbaI sites of pG3-CMP to generate pG3-C4 (Fig. 3). Plasmid DNA was electroporated into E. coli XL1-Blue according to manufacturer’s instructions (Stratagene, Inc.) and plated on LB agar containing 12.5 μg/ml chloramphenicol. The resulting colonies were pooled using 1 ml of LB medium. To determine that the pool size of each constructed library was large enough to have a 99% probability of containing the least probable sequence combination (e.g. Trp-Trp-Trp), the Poisson distribution was used (26). A 1/100 volume of the pooled colonies was used to prepare bacteriophage library stocks.

**Preparation of Phagemid Particles and Titering—**For packaging
phagemid. *E. coli* infected with the phagemid were packaged into 25 ml of 2YT containing 12.5 μg/ml chloramphenicol along with 1 × 107 VCS M13 helper phage (Stratagene, Inc.). After 15 min incubation at room temperature, the culture was grown shaking overnight at 37 °C. The bacteria were pelleted after overnight growth, and the supernatant was used to isolate phage particles. Previous studies from our laboratory (11) have shown that the β-lactamase variant E240K is expressed as an active enzyme from the expression vector pBG66. Based on these findings, the previously constructed β-lactamase mutants E240K and L113A/T114R in pBG66 were used to purify the corresponding β-lactamase enzymes (11, 27). For expression of the active β-lactamase single mutant A237T and the double mutant P107I/V108I, the expression vector pTP123 was used for panning (20). This vector was utilized since the β-lactamase variants from pG3-C4 could easily be cloned into the multi-cloning site of pTP123 and placed under the control of the inducible Ptrc promoter for increased β-lactamase expression if desired. The β-lactamase variants A237T and P107I/V108I were digested from the pG3-C4 vector with *Sac* I and *Xba* I to release the β-lactamase promoter and gene (Fig. 3). The fragments were gel-purified and ligated into the SallXbaI sites of the expression vector pTP123 to generate pGR34 and pGR36, respectively. The resulting plasmids were transformed into *E. coli* RB791 and selected on LB medium supplemented with 12.5 μg/ml chloramphenicol. The β-lactamase mutants were expressed under the β-lactamase constitutive promoter and purified as described with the pBG66 clones. The triple mutant L113A/T114R/E240K was constructed in pBG66 as described above and purified from *E. coli* RB791.

**Enzyme Kinetics**—The kinetic parameters of the β-lactamase mutant enzymes were determined with cephaloridine as a substrate using a 0.1-cm path length cuvette as described previously (28). Values reported are based on velocity measurements at 100, 200, 300, 400, 500, 600, 700, and 800 μM substrate concentrations.

**BLIP Inhibition Assay**—Inhibition assays were performed using the substrate cephaloridine as described previously with the following modifications (20). For the β-lactamase mutants L113A/T114R/E240K, E240K, and L113A/T114R/E240K, 0.1 μM β-lactamase was incubated with varying concentrations of 6× histidine-tagged BLIP. For the wild-type β-lactamase and β-lactamase mutants P107I/V108I and A237T, 1.0 μM β-lactamase was incubated with varying concentrations of the 6× histidine-tagged BLIP.

**RESULTS**

**Phage Display of β-Lactamase**—The crystal structure of the TEM-1 β-lactamase-BLIP complex shows that BLIP associates mainly with the 99–112 loop-helix and the 234–240 B3 β-strand lining the active site pocket of TEM-1 β-lactamase; however, the relative importance of these residues for binding BLIP remains unknown (21). In previous experiments by this laboratory (27) to determine the amino acid residues that are critical for the structure and function of TEM-1 β-lactamase, blocks of three contiguous codons were randomized to create a library containing all possible amino acid substitutions. Constructing a set of 88 individual libraries randomized the entire coding sequence of the gene (283 codons). Nine of these libraries, encompassing residues 99–114 and 232–240, covered the active site residues of TEM-1 β-lactamase; functional random mutants were selected from these libraries based on the ability to bind BLIP using monovalent phage display.

In order to display the β-lactamase variants from the random libraries on the surface of the M13 bacteriophage, the pG3-C4 phagemid was constructed (Fig. 3). This vector encodes a fusion of β-lactamase to the N terminus of the M13 gene III protein (gIIIp), which is transcribed by the constitutive β-lactamase promoter. An amber codon present between the β-lactamase gene and gene III allows for expression of the fusion protein in amber suppressor strains of *E. coli* and expression of only β-lactamase in non-amber suppressor strains (31). Because the vector encodes chloramphenicol resistance, bacteria harboring this vector can be selected on medium containing chloramphenicol.

**DNA Sequencing**—DNA sequencing of β-lactamase mutants was performed directly on polymerase chain reaction products of *bla*TEM amplified from single colonies (32). Oligonucleotides used for DNA sequencing were designed to prime specific sites within *bla*TEM.
Specific Enrichment of Randomized β-Lactamase Phage Libraries—In order to sample all amino acid substitutions in the loop-helix region and B3 β-strand of TEM-1 β-lactamase, each of the nine β-lactamase randomized libraries were inserted into the pG3-C4 vector to create the β-lactamase phage display libraries. To determine the β-lactamase residues important for binding with BLIP, phage from each library were selected based on their ability to bind immobilized BLIP. Three controls were used to show that the phage binding to the immobilized BLIP was dependent on the β-lactamase-BLIP interaction. First, phage displaying a library of β-lactamase mutants were panned on immobilized BSA in addition to the immobilized BLIP. Three controls were used to show that the phage binding to the immobilized BLIP was dependent on the β-lactamase-BLIP interaction. First, phage displaying a library of β-lactamase mutants were panned on immobilized BSA in addition to the immobilized BLIP. Three controls were used to show that the phage binding to the immobilized BLIP was dependent on the β-lactamase-BLIP interaction. First, phage displaying a library of β-lactamase mutants were panned on immobilized BSA in addition to the immobilized BLIP. After three rounds of binding and selection of the random library 112–114, over 1600-fold more β-lactamase phage bound to immobilized BLIP than to the BSA control (Fig. 4). These data indicate that the β-lactamase displaying phage can be rapidly enriched when panned against immobilized BLIP and that the enrichment is not due to nonspecific binding to BSA.

As a second control, phage displaying libraries of β-lactamase mutants were incubated with immobilized polyclonal anti-β-lactamase antibody. Since the polyclonal anti-β-lactamase antibody is capable of recognizing multiple epitopes on β-lactamase, it was expected that the majority of the displayed β-lactamase molecules would still bind the polyclonal antibody even if they contained substitutions in the BLIP-binding surface. After one round of panning of the 112–114 randomized library, 70-fold more phage bound the polyclonal anti-β-lactamase antibody than bound to BLIP (Fig. 4). However, after three rounds of panning only 5-fold more phage bound the antibody versus BLIP. Similar results were obtained with the remaining phage displaying random libraries (data not shown). These data indicate that the β-lactamase mutants from the libraries were efficiently displayed on the phage and provide further evidence that after several rounds of panning β-lactamase mutants that bind BLIP can be selected.

Finally, an internal control was used in which phage displaying β-lactamase libraries were incubated with an equal number of phage that did not display a protein in the presence of the immobilized targets BLIP, BSA, or polyclonal anti-β-lactamase

**Fig. 2.** Sequence results of randomized TEM-1 β-lactamase libraries after panning against immobilized BLIP. The eight randomized TEM-1 β-lactamase libraries are boxed. The wild-type TEM-1 β-lactamase sequence is shown above each library. Immediately below each residue number are the different amino acids that were identified at that sequence position after three rounds of panning against immobilized BLIP. Superscript numbers indicate the number of times each mutant appeared among the selected clones. For libraries 106–108 and 112–114 round 1 and round 2 results are shown below round 3 results as indicated. The consensus sequence was constructed from the results of Table I in which residues at each position shown were significantly enriched above their expected random frequency. The wild-type TEM-1 β-lactamase sequence is shown above the corresponding residue position with the consensus sequence residues shown below.
antibody. The pG3-SPT vector used to produce non-displaying phage is similar to the randomized β-lactamase phage display vector pG3-C4 except the chloramphenicol cassette is replaced with the spectinomycin cassette, and no fusion is made with gene III (29). This results in the production of phage that do not display a fusion protein and whose presence can be determined by spreading infected E. coli on agar plates containing spectinomycin. The advantage of this system is that the number of nonspecific phage interactions versus interactions dependent on β-lactamase can be determined by titering the phage recovered from a binding experiment on spectinomycin medium as well as chloramphenicol medium. After one round of panning library 112–114, 190-fold more β-lactamase-displaying phage than non-displaying phage were recovered after binding BLIP (Fig. 4). After three rounds of panning, 28,000-fold more β-lactamase-displaying phage bound BLIP compared with non-displaying phage. These data show that the majority of the binding interactions are specific for BLIP and β-lactamase and not due to nonspecific phage-protein interactions. Since similar numbers of pG3-SPT non-displaying phage were recovered from BSA and β-lactamase antibody binding experiments, it is believed that these numbers represent the background level of nonspecific protein interactions or trapping of phage by the beads.

Selecting for High Affinity β-Lactamase-BLIP Complexes—The residues in the 99–114 and 232–240 regions of TEM-1 β-lactamase that are most critical for binding BLIP were determined by screening each of the nine libraries covering these regions for the ability to bind tightly to BLIP. After three rounds of binding enrichment representative clones were sequenced (Fig. 2). At several positions a certain residue type was more prevalent among functional binding mutants than was expected from a random distribution of amino acids in the starting library (Table I). For example, after three rounds of binding enrichment on immobilized BLIP, 97% of the clones sequenced from the 112–114 random library contained a T114R substitution. This was 17 standard deviation units (17σ) above the expected frequency based on a random chance occurrence for Arg at this position in the library. Following a similar procedure for the remaining libraries, a consensus sequence for the loop-helix and B3 β-strand regions of TEM-1 β-lactamase was established for the mutants retaining the capacity to bind BLIP (Fig. 2).

Out of the 16 residues randomized in the 99–114 loop-helix region, seven positions exhibited a strong bias for the wild-type residue including Asp-101, Leu-102, Val-103, Ser-106, Pro-107, Thr-109, and His-112 (Fig. 2 and Table I). The results indicate that these residues are critical for tight binding with BLIP or for maintaining the structure and function of β-lactamase. At 4 of the 16 residues, the wild-type residue as well as one or two substitutions appear frequently among the selected clones. These include positions Gln-99, Glu-104, Tyr-105, and Val-108. In all cases except for Gln-99, non-wild-type substitutions are preferred (Table I). For example, Asn was favored over the aromatic side chain Tyr-105 by 3σ and Ile was favored over Val-108 by almost 6σ. For Glu-104, both Glu and Trp were distributed almost equally among the mutants (10.1σ and 10.9σ, respectively) indicating that both are consistent with binding of BLIP. The results suggest that these positions are
important for tight binding with BLIP; however, the sequence requirements are not as stringent since a limited number of substitutions are tolerated without dramatically affecting binding with BLIP. Three positions, Lys-111, Leu-113, and Thr-114, exhibited a bias for a non-wild-type residue. The preferred substitutions were K111T (9.6 $s$), L113A (21.2 $s$), and T114R (16.9 $s$) (Table I). The bias toward non-wild-type residues at these positions suggests that the substituted residues provide for a tighter binding complex between TEM-1 $\beta$-lactamase and BLIP compared with the wild-type residue. The two remaining residues, Asn-100 and Glu-110, had no strong bias for any BLIP compared with the wild-type residue. The two remaining positions, Thr-109 and Val-108, were equally represented and making up 66% of the population. Out of the five residues studied, the only position that did not exhibit a strong bias for the tight binding of BLIP was Thr-109; however, the sequence requirements are not as stringent since a limited number of substitutions are tolerated without dramatically affecting binding with BLIP. Three positions, Lys-111, Leu-113, and Thr-114, exhibited a bias for a non-wild-type residue. The preferred substitutions were K111T (9.6 $s$), L113A (21.2 $s$), and T114R (16.9 $s$) (Table I). The bias toward non-wild-type residues at these positions suggests that the substituted residues provide for a tighter binding complex between TEM-1 $\beta$-lactamase and BLIP compared with the wild-type residue. The two remaining residues, Asn-100 and Glu-110, had no strong bias for any particular residues. This suggests that these positions are not important for binding BLIP.

After the third round of panning the $\beta$-lactamase phage libraries 106–109 and 112–114, only one sequence was identified from all clones sequenced for each library. Library 106–109 converged on the amino acid sequence Ser-Pro-Val and library 112–114 converged on the sequence His-Ala-Arg. To verify that these results were not due to “siblinging” in which a single clone was amplified during the binding enrichment based on factors other than binding to the target molecule, the libraries were re-panned (31). After each round of panning, representative clones were sequenced to verify the diversity of the library. After the first round of panning library 106–109, 11 clones were sequenced, and 10 different amino acid sequences were identified, with only the mutant Ser-Pro-Ile being represented twice in the library (Fig. 2). Even after one round of panning a clear consensus for each position was seen in that 72% of the clones contained Ser at position 106, 54% contained Pro at position 107, and 100% contained a hydrophobic residue at position 108. Round two sequencing results identified four different clones, with both Ser-Pro-Val and Ser-Pro-Ile being equally represented and making up 66% of the population. After round three, the sequences converged on the wild-type Ser-Pro-Val sequence and the non-wild-type Ser-Pro-Ile sequence with various non-wild-type triplicate codons being represented in each case. These data indicated that during the initial panning of the library sibbing occurred and the Ser-Ile-Ile sequence overtook the phage population during the selection process. The re-panning and sequencing of library 112–114 revealed similar results as library 106–108 for the first two rounds of panning in that the library was initially diverse but quickly converged on certain residues at each position randomized (Fig. 2). By round three, the sequence converged on the same amino acid sequence, His-Ala-Arg, as that selected in the first set of binding experiments indicating that this sequence was strongly preferred for the tight binding of BLIP and was not a result of sibbing.

Three $\beta$-lactamase random libraries were used to study the B3 $\beta$-lactamase residues 232–240 (Fig. 2). Contamination of the library 232–240 by wild-type $\beta$-lactamase sequence prevented analysis of these positions. Out of the five residues studied, four exhibited a very strong bias for the wild-type residue among the sequences of functional mutants. These include residues Ser-235 (12.0 $s$), Gly-236 (14.9 $s$), Ala-237 (12.7 $s$), and Gly-238 (15.5 $s$) (note: TEM-1 $\beta$-lactamase has no residue 239 (33)) (Table I). In addition to Ala at position 237, Thr was also observed but to a much lesser degree (3.5 $s$) (Table I). This suggests that the B3 $\beta$-lactamase strands 235, 236, and 238 of TEM-1 $\beta$-lactamase are critical for the tight binding of BLIP. The only position that did not exhibit a strong bias for the wild-type residue among functional mutants was Gly-240.
than wild-type
predicted that the selected variants bind BLIP as tight or tighter
than wild-type. Among mutants selected for BLIP binding, it is as-
sumed that the selected variants bind BLIP as tight or tighter
than wild-type. This assumption was tested by purifying several of the selected 
- lactamase variants and determining the affinity for BLIP. The two loop-helix mutants 
studied were P107I/V108I and L113A/T114R. These mutants 
were strongly selected during the first set of phage binding experiments, suggesting a tight binding interaction with BLIP (data not shown). In addition, two B3 β-strand variants were studied, A237T and E240K.

The mutant β-lactamases to be studied were expressed independently of M13 gIIIp. Prior to determining the affinity of BLIP for the selected mutants, kinetic parameters were determined for each purified enzyme using the cephalosporin, cephaloridine, as a substrate (Table II). The kinetic parameters for the A237T and E240K enzymes agreed well with published values (11, 28). The P107I/V108I and L113A/T114R enzymes exhibited slightly lower catalytic efficiencies (kcat/Km) compared with wild-type β-lactamase for cephaloridine hydrolysis due to small decreases in both kcat and Km.

The equilibrium dissociation constants for the interaction between BLIP and the β-lactamase enzymes were determined using cephaloridine as a substrate in an assay developed previously (20). Wild-type or mutant β-lactamases were incubated in the presence of BLIP for 2 h. By monitoring the hydrolysis of cephaloridine added to the reaction after the incubation time, the concentration of free β-lactamase could be determined. Fitting the data obtained when incubating varying concentrations of wild-type β-lactamase in the presence of 1 nM BLIP resulted in a Kd of 0.22 nM (Table II), which compares favorably with previously reported data of 0.1 nM for BLIP purified from E. coli (20).

The enzyme containing the active site B3 β-strand substitution A237T was found to have a Kd for BLIP binding of 0.49 nM, whereas a Kd of 0.019 nM was determined for E240K (Table II). The Kd value obtained for the A237T enzyme suggests that the substitution interferes slightly with the binding of BLIP. This is consistent with the phage display data that indicated a strong bias for the wild-type Ala residue over the Thr residue among mutants selected for BLIP binding. The E240K enzyme binds BLIP 11-fold tighter than wild-type β-lactamase, indicating the positive charge or extended side chain at position 240 improves the interaction between β-lactamase and BLIP.

As stated above, in the first set of phage binding enrichments, an enzyme containing the P107I/V108I substitution in the loop-helix region predominated among selected mutants.
changes in the free energy of binding (34). Because the \( \beta \)-lactamase mutants A113L/T114R and E240K both exhibited increased binding affinity for BLIP, we sought to test whether the substitutions act additively by constructing the triple \( \beta \)-lactamase mutant A113L/T114R/E240K. The equilibrium dissociation constant of the triple \( \beta \)-lactamase mutant was determined as a \( K_i \) of 0.00042 nM (Fig. 5 and Table II). This binding was 550-fold tighter than binding by wild-type TEM-1 \( \beta \)-lactamase. Based on simple additivity, it was expected that the triple mutant would bind BLIP approximately 75-fold tighter than wild-type \( \beta \)-lactamase. Therefore, the A113L/T114R and E240K substitutions appear to act synergistically to produce a very tight binding interaction between \( \beta \)-lactamase and BLIP.

**DISCUSSION**

Over the past several years, maintaining the effectiveness of \( \beta \)-lactam antibiotics and \( \beta \)-lactamase inhibitors has become an increasing problem. BLIP has been shown to be a potent inhibitor of several class A \( \beta \)-lactamases and also exhibits inhibitory activity toward the penicillin-binding protein 5 of *Enterococcus faecalis* (19). Understanding the molecular interactions between BLIP and TEM-1 \( \beta \)-lactamase may aid the development of novel inhibitors of both \( \beta \)-lactamases and penicillin-binding proteins that are based on the structure of BLIP.

The crystal structure of TEM-1 \( \beta \)-lactamase complexed with BLIP indicates that over two-thirds of all contacts made by BLIP occur in the 99–112 loop-helix of TEM-1 \( \beta \)-lactamase, whereas more than 50% of the remaining contacts are made with the B3 \( \beta \)-strand residues 234–240 in the active site of the enzyme (21). The role of these residues in the binding of BLIP was determined using a set of eight \( \beta \)-lactamase random libraries that encompassed the loop-helix and B3 \( \beta \)-strand. The \( \beta \)-lactamase variants were displayed as fusion proteins to the gIIIp of M13. A newly constructed vector, pG3-C4, allows for low level constitutive expression of the \( \beta \)-lactamase-gIIIp fusion from the weak \( \beta \)-lactamase promoter. This low level expression reduces the potential toxicity of the gIIIp fusion and provides for monovalent display of the protein fusion from M13 phage (35).

The \( \beta \)-lactamase random libraries were used to construct a consensus sequence for the loop-helix and active site B3 \( \beta \)-strand regions showing the preferred residues at each position for tight binding with BLIP (Fig. 2). This consensus sequence differs from the wild-type \( \beta \)-lactamase sequence by as much as 52%, suggesting that the binding between TEM-1 \( \beta \)-lactamase and BLIP has not been optimized and can be improved. This is not surprising in that BLIP most likely did not evolve under selective pressure to bind TEM-1 \( \beta \)-lactamase since TEM-1 \( \beta \)-lactamase is prevalent in enteric bacteria, and BLIP is produced by a soil bacterium (18). In addition, TEM-1 \( \beta \)-lactamase is found only in the periplasmic space of Gram-negative bacteria and the Gram-negative outer membrane would prevent BLIP from reaching the periplasm. The biological target of BLIP is unknown; however, *S. clavuligerus* also produces a class A \( \beta \)-lactamase, which has been proposed to be the natural target of BLIP (36). The amino acid sequence in the loop-helix and B3 \( \beta \)-strand regions of the *S. clavuligerus* \( \beta \)-lactamase is 52% identical to the TEM-1 \( \beta \)-lactamase sequence and 52–66% identical to the consensus sequence depending on the selected consensus residues. Seven out of the 10 residues identified as critical in TEM-1 \( \beta \)-lactamase for binding BLIP are conserved in the *S. clavuligerus* \( \beta \)-lactamase including Asp-101, Ser-106, Pro-107, Thr-109, His-112, Gly-236, and Gly-238. The remaining critical TEM-1 \( \beta \)-lactamase residues Leu-102, Val-103, and Ser-235 consist of Val, Glu, and Thr, respectively, in the *S. clavuligerus* \( \beta \)-lactamase. Although a \( K_i \) for *S. clavuligerus* has not been determined, it has been reported that the *S. clavuligerus* \( \beta \)-lactamase is strongly inhibited in the presence of 5 \( \mu \)M BLIP. This is approximately 100-fold higher than the amount of BLIP required to strongly inhibit TEM-1 \( \beta \)-lactamase (36). These few amino acid differences may account in part for the reduced inhibitory effects of BLIP for the *S. clavuligerus* \( \beta \)-lactamase compared with that of TEM-1 \( \beta \)-lactamase.
Amino acid substitutions at Leu-113, Thr-114, and Glu-240 exhibited a bias for non-wild-type residues, which appear to improve the binding interaction between β-lactamase and BLIP. This is evident by the in vitro binding studies in which L113A/T114R and E240K β-lactamase variants were shown to bind BLIP 6- and 11-fold tighter than the wild-type β-lactamase, respectively (Fig. 5 and Table II). Examination of the BLIP-β-lactamase interface in the 113–114 region indicates that the T114R substitution could introduce interactions with Asp-68, Ser-69, or Tyr-115 of BLIP. Molecular modeling also suggests these new interactions could displace one or more ordered water molecules that are present at the BLIP-β-lactamase interface (21). Ordered water molecules at a protein-protein interface can fill in gaps between imperfectly packed regions (21, 37). Displacement of these water molecules by improved packing should result in tighter binding because of the more favorable entropy of the complex.

In contrast, the E240K/E240R substitution is not in a region of ordered water molecules. Rather, the Glu-240 residue makes van der Waals contacts with the BLIP residue Phe-142 (Fig. 6). BLIP Phe-142 is inserted in the active site pocket of TEM-1 β-lactamase and is critical for inhibition (20). Molecular modeling suggests that Arg or Lys at position 240 could increase the mutations A237T, G238S, and E240K (20). These findings suggest that BLIP is not only a potent inhibitor of TEM-1 β-lactamase but may also be a potent inhibitor of many of the 61 currently known TEM-based β-lactamases.

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