Contributions of Aspartate 49 and Phenylalanine 142 Residues of a Tight Binding Inhibitory Protein of β-Lactamases*

(Received for publication, October 5, 1998, and in revised form, November 13, 1998)

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β-Lactamases are bacterial enzymes that hydrolyze β-lactam antibiotics to render them inactive. The β-lactamase inhibitor protein (BLIP) of Streptomyces clavuligerus, is a potent inhibitor of several β-lactamases, including the TEM-1 enzyme (Kᵢ = 0.6 nM). Evidence from the TEM-1/BLIP co-crystal suggests that two BLIP residues, Asp-49 and Phe-142, mimic interactions made by penicillin G when bound in the active site of TEM-1. To determine the importance of these two residues, a heterologous expression system for BLIP was established in Escherichia coli. Site-directed mutagenesis was used to change Asp-49 and Phe-142 to alanine, and inhibition constants (Kᵢ) for both mutants were determined. Each mutation increases the Kᵢ for BLIP inhibition of TEM-1 β-lactamase approximately 100-fold. To address how these two positions effect the specificity of β-lactamase binding, Kᵢ values were determined for the interaction of wild-type BLIP, as well as the D49A and F142A mutants, with two extended spectrum β-lactamases (the G238S and the E104K TEM variants). Positions 104 and 238 are located in the BLIP/β-lactamase interface. Interestingly, the three BLIP proteins inhibited the G238S β-lactamase mutant to the same degree that they inhibited TEM-1. However, wild-type BLIP has a higher Kᵢ for the E104K β-lactamase mutant, suggesting that interactions between BLIP and β-lactamase residue Glu-104 are important for wild-type levels of BLIP inhibition.

β-Lactamases are bacterial enzymes that confer resistance to β-lactam antibiotics, which include the penicillins and cephalosporins. These drugs act by inhibiting bacterial penicillin binding proteins (PBP) that are essential for the synthesis of the bacterial peptidoglycan layer (1). Several resistance mechanisms have evolved in bacteria to protect them from the lethal effects of β-lactam antibiotics.

β-Lactamase imparts resistance to the β-lactams by hydrolyzing the amide bond in the four-membered β-lactam ring (2). Genes encoding β-lactamases may be found on plasmids, transposons, and on the bacterial chromosome (2–6). There are four classes of β-lactamases (classes A–D), categorized by their primary sequence homology. β-Lactamase-mediated resistance has increased because of selective pressure from widespread use of β-lactam antibiotics and is now a serious threat to antibiotic therapy (7).

TEM-1 β-lactamase, encoded by the blaTEM-1 gene, is among the most prevalent plasmid-encoded β-lactamases found in Gram-negative bacteria (8). The name TEM is derived from the name of the patient carrying the pathogen from which the enzyme was isolated (9). It efficiently hydrolyzes penicillins and most cephalosporins (2). However, it is a poor catalyst for hydrolysis of the newer third-generation cephalosporins, such as ceftazidime, that were designed to circumvent TEM-1-mediated inactivation.

In addition to developing new drugs that are unable to be cleaved by β-lactamase, another method to counter the hydrolytic activity of this enzyme has been to administer β-lactamase inhibitors, such as sulbactam and clavulanic acid. Use of an inhibitor along with an existing β-lactam antibiotic is an effective means to treat various β-lactamase producing bacterial pathogens (10). Clavulanic acid was originally found as a metabolite of the Gram-positive soil bacterium Streptomyces clavuligerus and, today, is one of the most commonly used β-lactamase inhibitors.

β-Lactamase-mediated resistance has been exacerbated by the fact that specific mutations in TEM-1 β-lactamase enable the enzyme to hydrolyze the newer third-generation antibiotics (11, 12). These evolved β-lactamases, called extended spectrum β-lactamases (ESBLs), provide clinically relevant levels of resistance to even the most recently developed β-lactams. Furthermore, other mutants have been found with substitutions that allow β-lactamase to avoid inactivation by the β-lactamase inhibitors (13). A similar result has been observed with SHV-1 β-lactamase, another class A enzyme that is 68% identical to TEM-1 β-lactamase (7). Recently, a β-lactamase mutant (TEM-50) has been recovered from clinical isolates with both types of mutations, enabling β-lactamase to hydrolyze extended spectrum antibiotics and avoid inactivation by inhibitors (14).

In 1990, the β-lactamase inhibitor protein (BLIP) was isolated from S. clavuligerus culture supernatants (15). BLIP is a 165-amino acid protein in its secreted form and is a potent inhibitor of TEM-1 β-lactamase (Kᵢ = 0.6 nM) (16). BLIP is able to inhibit several β-lactamases, as well as weakly inhibit a penicillin-binding protein (PBP) from Enterococcus faecalis (16). The DNA sequence and crystal structure of BLIP have been determined, as well as the co-crystal with TEM-1 β-lactamase (16, 17).

The BLIP crystal structure shows that the protein has a novel fold with two similar domains. The BLIP mechanism of inhibition appears to be two-fold. At 2636 Å², the surface area of the enzyme/inhibitor interface is one of the largest known for protein/protein complexes as BLIP essentially clamps over the active site of β-lactamase (17). In addition, an aspartic acid

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* This work was supported in part by National Institutes of Health Grant AI32956. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: PBP, penicillin-binding protein; ESBL, extended spectrum β-lactamase; BLIP, β-lactamase inhibitor protein; PenG, penicillin G; 6XHis, 6-histidine; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
residue at position 49 of BLIP aligns itself in the active site pocket and forms strong hydrogen bonds with four catalytic residues of β-lactamase. Furthermore, a phenylalanine at position 142 of BLIP appears to mimic the benzyl group of the β-lactam antibiotic penicillin G (PenG) and further stabilizes the inhibitory complex (17). This result is potent inhibition of TEM-1 β-lactamase.

The ongoing problem of targeting bacteria with antimicrobial agents able to circumvent ESBL-mediated antibiotic inactivation creates a need for new potent inhibitors of β-lactamases. The ability to engineer BLIP to tightly bind the ESBLs and PBPs would aid in the design of effective new antimicrobials. New protein-protein interactions found to be effective in inhibiting β-lactamases and PBPs could be duplicated by a peptide inhibitor or synthetic compound designed to mimic the interactions. It would be easier to perform such studies with BLIP if the inhibitor could be expressed in a more manageable genetic system. This report describes the efficient expression of functional BLIP in E. coli and the use of this system to determine the importance of residues Asp-49 and Phe-142 in BLIP Inhibition.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—E. coli XLI-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, (F’ proAB, lacI q lacZD15, Tn10tet(r)) was used to propagate plasmid DNA (Stratagene, Inc.). E. coli RB791 (= strain W3110 lacP L8) was used to express BLIP and the D49A and F142A BLIP mutants (18, 19). Plasmid pTP123 is a cpm’ amp’ derivative of pTrc 99A (Amersham Pharmacia Biotech) (see Fig. 1). It was created by ligating the HindIII cassette from pKBPII into BsaI- digested pTrc 99A (20). The BsaI site was filled in using the Klenow fragment of DNA Polymerase I prior to ligation. This cloning step inserts a chloramphenicol acetyltransferase (cat) gene into the rrnBT1 T2 transcriptional terminators and part of the β-lactamase gene encoded by pTrc 99A. As a result, functional β-lactamase is not expressed, and potential difficulty in BLIP purification because of binding of endogenous β-lactamase is avoided. The cat gene in pTP123 is in the same orientation as the trc promoter.

BLIP Cloning and PCR Mutagenesis—A 6-histidine (6XHis) tag was first inserted between the β-lactamase signal sequence and the BLIP coding sequence of pG3-BLIP (21) by overlapping PCR mutagenesis (22). The internal mutagenic PCR primer sequences are: BLIPHIS-1 (a top strand primer), 5’-CACCCACCACCATCCACCGGGGGGTGATG-3’; and BLIPHIS-2 (a bottom strand primer), 5’-CGGCTTGTTGGTTGGTTGGTTTGGTTTGGTGAGCAGGACAAAAAACGGG- AAGCCA-3’. The external PCR primers used to amplify the construct are: PD-bla1 (top strand, N-terminal), 5’-CGGGAGGCTCGTTTCTTA- GACGTACCAGTGCCC-3’; and MALBLI-2 (bottom strand C-terminal), 5’-GGGAAAATCTAGATTATACAAGGTCCCACTGCCG-3’. A SacI site in PD-bla1 and a XbaI site in MALBLI-2 allowed the PCR product to be cloned into SacI- and XbaI-digested pTP123 following treatment of the vector with calf intestinal alkaline phosphatase. The final SacI/XbaI fragment contains, from 5’ to 3’, the TEM-1 β-lactamase constitutive promoter, the TEM-1 β-lactamase signal sequence, and a 6XHis tag at the N terminus of the His-BLIP construct.

FIG. 1. Plasmid maps for pTP123 and pGR32. The BLIP cassette, possessing an N-terminal His-tagged BLIP gene, under control of the β-lactamase promoter, was inserted into SacI/XbaI-digested pTP123. This construct also features the β-lactamase signal sequence fused to the N terminus of the His-BLIP construct.

A 6-histidine (6XHis) tag was encoded by pTrc 99A. As a result, functional BLIP was expressed, and potential difficulty in BLIP purification because of binding of endogenous β-lactamase is avoided. The cat gene in pTP123 is in the same orientation as the trc promoter.

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PCR Mutagenesis—Construction of the BLIP D49A and F142A mutants was accomplished by overlapping PCR mutagenesis (22). PD-bla1 and MALBLI-2 were used as external primers in these mutagenesis reactions. The internal top and bottom primers for constructing the D49A mutant are: BP-D49A (top), 5’-CGGGGGCAGCGGCAAGGGCCT- TTACTACGCCCTACGCCACCC-3’; and D49A (bottom), 5’-GGTGCGGGCT- AGGGCTAGTAGGCCCTCGGCGCTGCCCCC-3’. The internal top and bottom primers for constructing the F142A mutant are: BP-F142A (top), 5’-GGTTAATCGTCCAGGGTGGTTCTTACCAGGAGTCCGCGCA-3’; and F142A-BOT (bottom), 5’-GTCGGCCGAGCTTGTTAAGCCCCGTCGCAAGATACC-3’. PD-bla1 and MALBLI-2 were used to amplify the full-size mutagenized product. Both mutagenic PCR products were cloned into SacI/XbaI-digested and calf intestinal alkaline phosphatase-treated pTP123. The plasmid containing the D49A mutant was named pJP128, and the plasmid containing the F142A mutant was named pJP129. The DNA sequence of each mutant was confirmed by the dyeoxy chain-termination method.

BLIP and β-Lactamase Expression and Purification—Plasmids pGR32, pJP128, and pJP129 were transformed into E. coli RB791 by electroporation. An overnight culture of each was grown shaking in 40 ml of Luria-Bertani (LB) medium at 37 °C in the presence of 12.5 μg/ml chloramphenicol. The 40 ml of overnight culture was used to inoculate 2 liters of LB medium containing 12.5 μg/ml chloramphenicol. The bacteria were then grown shaking at 25 °C until A600nm = 1.2. For induction of BLIP, 3 mM IPTG was added to each culture, and the cultures were then allowed to grow an additional 5 h.

Following the 5-h induction, the cells were pelleted and resuspended...
in 15 ml of sonication buffer (20 mM Tris-HCl (pH 8.0) and 500 mM NaCl). The cells were then sonicated in two batches, and insoluble material was pelleted by centrifugation. The soluble protein in the supernatant was purified over a 4-mL TALON column (CLONTECH) according to the manufacturer instructions. A 4 mM imidazole wash step was utilized to remove protein from the column which bound less tightly than the His-tagged BLIP. BLIP was eluted using an elution buffer consisting of 50 mM imidazole added to the sonication buffer (pH 8.0). Fractions were examined by SDS-PAGE to estimate purity and yield (Fig. 2). Approximately 500 μg of >90% pure BLIP could be isolated for every two liters of culture using this strategy. Quantitative amino acid analysis was performed to calibrate a Bradford assay for determining BLIP and β-lactamase concentrations (23).

Wild-type β-lactamase and the G238S and E104K extended spectrum mutants were expressed and purified as described previously (24). The location of the two extended spectrum mutations, with respect to Asp-49 and Phe-142 of BLIP, is shown in Fig. 3.

BLIP Inhibition Assay—Varying concentrations of BLIP were incubated with 1 mM β-lactamase for 2 h at 25 °C. 2 nM of the β-lactamase were used in the G238S studies. The enzyme-inhibitor incubation was done in 0.05 M phosphate buffer (pH 7.0) containing 1 mg/ml bovine serum albumin. Following the 2-h incubation, which is sufficient time to achieve binding equilibrium, cephaloridine concentration is found in many extended spectrum enzymes (26). This single mutation increases the catalytic efficiency of β-lactamase for the third generation cephalosporins ceftazidime and cefotaxime approximately 70- and 40-fold, respectively (27). The E104K mutation, likewise, has been found in many extended spectrum β-lactamase variants (26). This mutation increases the catalytic efficiency of β-lactamase approximately 50-fold for ceftazidime and 10-fold for cefotaxime (28). Both Gly-238 and Glu-104 are located at the binding interface of BLIP and β-lactamase (17). Wild-type BLIP was found to have a Ki of 0.07 nM for G238S, and a Ki of 14 nM for E104K (Figs. 5 and 6, Table I). These values suggest that the G238S mutation has little effect on the binding of wild-type BLIP to β-lactamase, whereas the E104K mutation interferes with binding in such a way that the Ki increases 1000-fold.

Mutant BLIP Binding—The x-ray structure of the BLIP-TEM-1 β-lactamase complex shows that BLIP residues Asp-49 and Phe-142 mimic portions of the β-lactam PenG when bound to β-lactamase (17). This structural mimicry suggests that these residues maintain important interactions in the inhibitor complex. To determine the contribution of these amino acids to inhibition of TEM-1 β-lactamase and extended spectrum-hydrolyzing β-lactamases, the D49A and F142A mutants were constructed. The Ki of each was determined with TEM-1, E104K, and G238S β-lactamases (Figs. 4–6; Table I).

Both the D49A and F142A mutants exhibited an approximately 100–300-fold increase in Ki compared with wild-type BLIP inhibition of TEM-1 β-lactamase. The D49A mutant inhibits TEM-1 with a Ki of 8.3 nM, whereas the F142A mutant inhibits with a Ki of 33 nM. To ascertain whether these mutations affect inhibition in an additive manner, the D49A/F142A double mutant was constructed. However, we were unable to express the double mutant under the same strategy used with wild-type BLIP and the other single amino acid mutants.

The interactions of the wild-type, D49A, and F142A BLIP inhibitors with the ESBLs show that BLIP binds G238S with similar strength as that of wild-type TEM-1, but binds E104K weakly. The Ki values found for D49A and F142A mutants with G238S β-lactamase were similar to the Ki values found for the BLIP mutants binding TEM-1. The D49A BLIP mutant inhibited G238S with a Ki of 9.4 nM. Therefore, as with TEM-1, the D49A mutation reduced inhibition 100-fold. Likewise, the F142A BLIP bound G238S β-lactamase approximately 800-fold weaker, with a Ki of 55 nM. The fact that these two substitutions in BLIP have a similar effect on the Ki values for the BLIP was incubated with a target for 2 h, which is sufficient time to achieve equilibrium. After the 2-h incubation, cephaloridine (at a concentration 10-fold less than the cephaloridine Km for the β-lactamase being tested) was added. Monitoring the hydrolysis of cephaloridine at a concentration below Km allowed the concentration of uninhibited β-lactamase to be determined without shifting the binding equilibrium. The concentration of free β-lactamase was calculated from the cephaloridine activity in the presence of a given quantity of BLIP, the cephaloridine activity in the absence of BLIP, and the known molar concentration of β-lactamase being used. Fitting the data obtained when incubating varying concentrations of wild-type, His-tagged BLIP with 1 nM TEM-1 β-lactamase resulted in a Ki of 0.11 nM (Fig. 4, Table I). This value compares reasonably well with the previously reported value of 0.6 nM found with BLIP purified from S. clavuligerus (16) and suggests that the N-terminal 6XHis tag has little effect on the binding of the inhibitor to the TEM-1 enzyme.

To determine whether wild-type BLIP has similar affinity for extended spectrum β-lactamases, the Ki of BLIP for two representative ESBLs was determined. The G238S β-lactamase mutation is found in many extended spectrum enzymes (26). This single mutation increases the catalytic efficiency of β-lactamase for the third generation cephalosporins ceftazidime and cefotaxime approximately 70- and 40-fold, respectively (27). Asp-49 and Phe-142 mimic portions of the β-lactam PenG when bound to β-lactamase (17). This structural mimicry suggests that these residues maintain important interactions in the inhibitor complex. To determine the contribution of these amino acids to inhibition of TEM-1 β-lactamase and extended spectrum-hydrolyzing β-lactamases, the D49A and F142A mutants were constructed. The Ki of each was determined with TEM-1, E104K, and G238S β-lactamases (Figs. 4–6; Table I).
TEM-1 and G238S \(\beta\)-lactamases suggests that BLIP inhibits G238S in much the same way it inhibits TEM-1.

Further experiments showed that Asp-49 and Phe-142 do not contribute to the binding of E104K \(\beta\)-lactamase as they do to the binding of TEM-1 and G238S. The \(K_i\) of BLIP D49A with E104K \(\beta\)-lactamase is 1.5 \(\mu\)M, which is only a 10-fold increase compared with that of the wild-type BLIP interaction with E104K. This suggests that BLIP residue Asp-49 is not as critical to inhibition of E104K \(\beta\)-lactamase as it is to the other enzymes tested. This observation is even more pronounced with the BLIP F142A substitution in that there was little change from the wild-type BLIP \(K_i\) with the BLIP F142A mutant inhibiting E104K (\(K_i\) (F142A) \(= 240 \mu\)M). Therefore, in contrast to their effect on TEM-1 and G238S binding, the D49A and F142A substitutions do not have as detrimental an effect on the \(K_i\) for E104K \(\beta\)-lactamase.

**BLIP Binding to SHV-1 \(\beta\)-Lactamase—**The amino acid sequence of SHV-1 is 68% identical to TEM-1 \(\beta\)-lactamase (7). How this similarity corresponds to structure homology is unknown as the crystal structure of SHV-1 \(\beta\)-lactamase has not yet been solved. Both the TEM-1 and SHV-1 enzymes hydrolyze a similar profile of penicillins and cephalosporins. It is not clear whether the homology between the two enzymes implies...
that BLIP should inhibit both equally well. It may be that even slight differences in the three-dimensional structure of SHV-1 compared with TEM-1 would effect BLIP binding considerably. These issues were addressed by performing an additional inhibitory assay with wild-type BLIP and SHV-1 \( \beta \)-lactamase. SHV-1 was purified to greater than 90% homogeneity (data not shown) and was bound to increasing concentrations of wild-type BLIP. The \( K_i \) of BLIP for SHV-1 was found to be 1.0 \( \text{mM} \), 9,000-fold higher than what was found for TEM-1 (Fig. 7, Table I). Therefore, the high degree of identity between these enzymes does not translate to similar binding properties by BLIP.

**DISCUSSION**

The development of novel inhibitors for \( \beta \)-lactamases as well as penicillin-binding proteins would provide new options for the treatment of bacterial infections. As \( \beta \)-lactamases are believed to have evolved from PBPs, it is conceivable that minor changes in the structure of BLIP could enable it to bind and inhibit PBPs (29). It has already been observed that wild-type BLIP weakly inhibits PBP5 from *Enterococcus* (16). Understanding how the amino acid sequence of BLIP encodes its tight binding affinity for certain \( \beta \)-lactamases, and its weaker affinity for PBPs, would facilitate the development of novel inhibitors with potent activity for the ESBLs and for the PBPs.

The crystal structure of the BLIP-TEM-1 complex suggests that two BLIP amino acids, Asp-49 and Phe-142, are critical for
the inhibition of TEM-1 through interactions in the active site pocket (17). In addition, the BLIP amino acids that bind the 99–112 loop of β-lactamase (Ser-35, Phe-36, Ser-39, His-41, His-45, Ala-47, Gly-48, Tyr-50, Tyr-53, Ser-71, Glu-73, Lys-74, Ser-113, Gly-141, Tyr-143, Trp-150, Trp-112, Arg-160, Trp-162) represent the majority of the surface interactions between the two proteins (2636 Å² is buried at the intermolecular surface) (17). These residues are the primary candidates for mutational analysis to distinguish whether “hot spots” consisting of a subset of these amino acids are involved in specificity and binding or if all of these residues contribute to the binding of BLIP to TEM-1 β-lactamase.

The first step in being able to identify amino acids important for BLIP specificity and inhibitory activity is to develop an expression system to produce functional BLIP. BLIP expressed in its native S. clavuligerus produces large quantities of protein, whereas expression in another Streptomyces species, Streptomyces lividans, produces limited quantities of BLIP (15, 30). Successful expression of soluble BLIP in E. coli would facilitate the study of BLIP mutants and would also allow protein engineering techniques to be performed.

Histidine-tagged proteins are able to be purified in a relatively simple manner, while usually maintaining the native activity of the tagged protein. Therefore, an expression system centered around an N-terminal 6XHis-tagged BLIP was constructed. Expression is directed by the inducible trc promoter, and a cat gene is inserted into the β-lactamase gene of the plasmid to avoid possible complex formation during purification of BLIP (Fig. 1). This system enabled BLIP to be purified to >90% homogeneity in one step (Fig. 2). Wild-type His-tagged BLIP was found to have a $K_i$ of 0.11 nM. This value is slightly lower than the previously calculated value of 0.6 nM for BLIP isolated from S. clavuligerus (16). This difference could be attributed to the manner in which the $K_i$ was calculated, and confirms that the N-terminal His tag has no effect on BLIP binding.

Once expression of functional BLIP was achieved, the roles of BLIP residues in the inhibition of different targets could be determined. The crystal structure of BLIP with TEM-1 β-lactamase shows that Asp-49 of BLIP makes strong hydrogen bond contacts with four conserved residues in the TEM-1 active site pocket: Ser-130, Lys-234, Ser-235, and Arg-244 (17). These four amino acids are involved in the binding and catalysis of β-lactam antibiotics and are conserved in all class A β-lactamases. Mutation of the aspartic acid to an alanine removes the carboxylate moiety that serves as a hydrogen bond acceptor for the four active site TEM-1 residues. Elimination of the carboxylate reduced the inhibitory activity of BLIP approximately 100-fold, indicating residue Asp-49 does make an important contribution to BLIP inhibition of TEM-1 β-lactamase.

The crystal structure also leads to the prediction that Phe-142 is also important for inhibition of TEM-1 β-lactamase (17). Phe-142 is in contact with β-lactamase residues Glu-104, Tyr-105, Asn-170, Ala-237, Gly-238, and Glu-240 in the complex. As in the case of Asp-49, most of these residues are either conserved in class A β-lactamases or are involved in catalysis. It was also found that in other protein complexes, such as between human growth hormone and its receptor, the most critical interactions are hydrophobic (31). Therefore, the contribu-

![Fig. 7. Determination of wild-type BLIP $K_i$ for SHV-1 β-lactamase. BLIP inhibitory activity is expressed as the remaining concentration of free β-lactamase at varying inhibitor concentrations. SHV-1 concentration is 1 nM, and the cephaloridine concentration is 70 μM for all experiments. The apparent $K_i$ was determined as in Fig. 4.](image-url)
Gly-238 of TEM-1 is by Phe-142. The fact that no change in the inhibition profile was observed between the β-lactamase enzymes suggests that this contact between Gly-238 and Phe-142 is not critical for BLIP binding and inhibition. If this interaction played a role in BLIP inhibition, then replacement of the glycine side-chain at position 238 of TEM-1 would have resulted in an increased $K_i$ with wild-type BLIP. However, the possibility that the substituted serine at position 238 makes new interactions with BLIP, and corrects exactly for the loss of interaction with Phe-142, cannot be excluded.

In contrast to BLIP binding to G238S, significant changes in the inhibitory profile were observed when E104K was used as the target β-lactamase. Wild-type BLIP inhibited E104K approximately 1000-fold worse than for TEM-1, suggesting that the interactions made between BLIP and Glu-104 are critical for wild-type levels of activity. This result indicates that some, or all, of the BLIP residues interacting with Glu-104 (BLIP-Glu-73, Lys-74, Phe-142, and Tyr-143) are making important interactions. However, results from the BLIP F142A/β-lactamase E104K binding experiment suggest that the lysine substitution at position 104 of β-lactamase disrupts critical interactions made between Phe-142 of BLIP. This disruption of the BLIP-Phe-142 interaction with β-lactamase results in the pronounced loss of inhibition observed with the E104K enzyme.

It is apparent from this study that while BLIP residues Asp-49 and Phe-142 are critical for wild-type BLIP inhibitory levels, the D49A and F142A variants still bind TEM-1 β-lactamase with nanomolar affinity. Therefore, other interactions must also contribute to the strong levels of inhibition observed. The identification of the epitopes responsible for the remaining binding energy will facilitate the engineering of tighter, smaller inhibitors for these β-lactamases.

Determination of the $K_i$ of BLIP with SHV-1 β-lactamase shows that even though TEM-1 and SHV-1 are both class A β-lactamases and are 68% identical, the interactions which make BLIP a tight inhibitor of TEM-1 are not conserved with SHV-1. Although no crystal structure is available for SHV-1, the level of identity between TEM-1 and SHV-1 suggests that both enzymes share a similar protein fold. However, the fact that BLIP is a poorer inhibitor of SHV-1 shows that small differences between the β-lactamases are significant with respect to BLIP binding.

Acknowledgments—We thank Natalie Strynadka and Michael N. G. James for the BLIP-TEM-1 β-lactamase crystal coordinates. We also thank Dr. Wanzhi Huang for technical assistance.

REFERENCES

1. Tomasz, A. (1979) Annu. Rev. Microbiol. 33, 113–137
2. Bush, K., Jacoby, G. A., and Medeiros, A. (1995) Antimicrob. Agents Chemother. 39, 1211–1233
3. Ambler, R. P. (1980) Philos. Trans. R. Soc. Lond. B Biol. Sci. 289, 321–331
4. Joris, B., Ghysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Freire, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C., and Knox, J. R. (1988) Biochem. J. 250, 313–324
5. Matthew, M. (1979) J. Antimicrob. Chemother. 5, 349–358
6. Sanders, C. C. (1992) Clin. Infect. Dis. 14, 1089–1099
7. Petrosoin, J., Cantu, C., III, and Palzkill, T. (1998) Trends Microbiol. 6, 323–327
8. Wiedemann, B., Kliebe, C., and Kresken, M. (1989) J. Antimicrob. Chemother. 24, 1–24
9. Jacoby, G. A. (1994) Trends Microbiol. 2, 357–360
10. Parker, R. H., and Eggleson, M. (1987) Infect. Control 8, 36–40
11. Medeiros, A. A. (1997) Clin. Infect. Dis. 24, S19–S45
12. Jacoby, G. A., and Medeiros, A. A. (1991) Antimicrob. Agents and Chemother. 35, 1697–1704
13. Saves, I., Barlet-Schiltz, O., Swaren, P., Lefevre, F., Masson, J.-M., Preme, J.-C., and Samama, J.-P. (1995) J. Biol. Chem. 270, 18240–18245
14. Sirot, D., Recule, C., Chaibi, E. B., Bret, L., Croize, J., Chanal-Claris, C., Labia, R., and Sirot, J. (1997) Antimicrob. Agents Chemother. 41, 1322–1325
15. Doran, J. L., Leskiw, B. K., Aippersbach, S., and Jensen, S. E. (1990) J. Bacteriol. 172, 4999–4918
16. Strynadka, N. C. J., Jensen, S. E., Johns, K., Blanchard, H., Page, M., Matagne, A., Frere, J.-M., and James, M. N. G. (1994) Nature 368, 657–660
17. Strynadka, N. C. J., Jensen, S. E., Alzari, P. M., and James, M. N. G. (1996) Nat. Struct. Biol. 3, 290–297
18. Brent, R., and Ptashne, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4204–4208
19. Amann, E., Brosius, J., and Ptashne, M. (1983) Gene (Amst.) 25, 167–178
20. Reece, K. S., and Phillips, G. J. (1995) Gene (Amst.) 165, 141–142
21. Huang, W., Petrosoin, J., and Palzkill, T. (1998) Antimicrob. Agents Chemother. 42, 2893–2897
22. Ho, S. N., Hunt, H. D., Horton, R. H., Muller, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Cantu, C., III, Huang, W., and Palzkill, T. (1996) J. Biol. Chem. 271, 22538–22545
25. Dennis, M. S., and Lazarus, R. A. (1994) J. Biol. Chem. 269, 22129–22136
26. Du Bois, S. K., Marriott, M. S., and Amyes, S. G. B. (1995) J. Antimicrob. Chemother. 35, 7–22
27. Venkatachalam, K. V., Huang, W., Laffèco, M., and Palzkill, T. (1994) J. Biol. Chem. 269, 23444–23450
28. Pradakar, A. S., Petrich, A. K., Leskiw, B. K., Aidoo, K. A., and Jensen, S. E. (1994) Gene (Amst.) 144, 31–36
29. Clackson, T., and Wells, J. A. (1995) Science 267, 383–386
