The hydrolysis of β-lactam antibiotics by class A β-lactamases is a common cause of bacterial resistance to these agents. The β-lactamase inhibitory protein (BLIP) is able to bind and inhibit several class A β-lactamases, including TEM-1 β-lactamase and SME-1 β-lactamase. Although the TEM-1 and SME-1 enzymes share 33% amino acid sequence identity and a similar fold, they differ substantially in surface electrostatic properties and the conformation of a loop-helix region that BLIP binds. Alanine-scanning mutagenesis was performed to identify the residues on BLIP that contribute to its binding affinity for each of these enzymes. The results indicate that the sequence requirements for binding are similar for both enzymes with most of the binding free energy provided by two patches of aromatic residues on the surface of BLIP. Polar residues such as several serines in the interface do not make significant contributions to affinity for either enzyme. In addition, the specificity of binding is significantly altered by mutation of two charged residues, Glu73 and Lys74, that are buried in the structure of the TEM-1-BLIP complex as well as by residues located on two loops that insert into the active site pocket. Based on the results, a E73A/Y50A double mutant was constructed that exhibited a 220,000-fold change in binding specificity for the TEM-1 versus SME-1 enzymes.

Protein-protein interactions play a central role in many cellular processes, such as signal transduction, the immune response, and biochemical regulation of enzyme function. Engineering of protein-protein interactions or the rational design of drugs that disrupt interactions requires an understanding of the physical basis of affinity and specificity within an interface (1). Protein-protein interfaces are commonly large (>900 Å²) and exhibit good shape and electrostatic complementarity (2–5). Hydrophobic patches on the surface of one protein commonly pack against hydrophobic patches on the other protein, whereas charged residues also commonly match across an interface. Alanine-scanning mutagenesis has been used to experimentally test the contributions of individual residues to protein interactions (6, 7). Studies of several interfaces suggest that functional epitopes determined by mutagenesis are more restricted than structural epitopes revealed by x-ray crystallography (8). The presence of a small subset of “hotspot” residues that contribute significantly to the binding free energy of a complex may be a general feature of protein-protein interfaces (3). Based on current understanding, however, it is not possible to reliably predict the energetic contributions of individual residues based on the structure of a protein-protein interface (5). Therefore, additional structure-function studies are required to generate a detailed understanding of the critical components of protein-protein interfaces.

β-Lactam antibiotics such as the penicillins and cephalosporins are among the most frequently used antimicrobial agents. Resistance to these drugs is most commonly due to the action of β-lactamase enzymes. β-Lactamases catalyze the hydrolysis of the amide bond in the β-lactam ring to create an ineffective antimicrobial (9). There are four classes (A–D) of β-lactamases based on primary sequence homology (10). TEM-1 and SME-1 are class A β-lactamases that are found in Gram-negative bacteria. These enzymes are clinically significant because of their ability to confer β-lactam antibiotic resistance, which has become a serious threat for human health. Both enzymes are able to hydrolyze most of the penicillins and early cephalosporins but not third generation cephalosporins. SME-1 has a wider substrate spectrum than TEM-1 in that it catalyzes the hydrolysis of carbapenem antibiotics (11). The TEM-1 and SME-1 β-lactamases have been solved and indicate the enzymes possess a similar fold (12, 13). The charge characteristics of TEM-1 and SME-1 are substantially different, however, with pI values of 5.4 and 9.5, respectively (11).

The β-lactamase inhibitory protein (BLIP) is a 165-amino acid protein produced by Streptomyces clavuligerus that has been shown to be a potent inhibitor of class A β-lactamases including TEM-1 (14–16). The x-ray structure of BLIP reveals that it is a flat molecule composed of a tandem repeat of a 76-amino acid domain (16). The two domains form a concave surface that is lined with charged, polar residues such as serine and tyrosine. However, there are also three tryptophan and two phenylalanine residues that contribute to two hydrophobic patches on the concave surface. The co-crystal structure of BLIP with TEM-1 β-lactamase indicates that BLIP uses the large (2636 Å²) concave surface to clamp over a protruding loop and helix region of TEM-1 (residues 99–114) (17). In addition, at the periphery of the interface, two loops from BLIP insert into the active pocket of TEM-1 to block substrate binding (17). BLIP also contains three charged residues on its...
The coding sequences for these mutants overlap with MALbli-2 primer. Therefore, these constructions were performed by one-step PCR with the PBha-1 primer and modified Malbli-2 primers as described in Experimental Procedures.

In this study, alamine-scanning mutagenesis has been performed to determine the functional epitope of BLIP for binding two class A β-lactamases from Gram-negative bacteria. Alamine-scanning mutagenesis is a powerful tool for understanding the contributions of the side chains of individual residues to binding affinity (6, 7, 19, 20). The apparent binding constants \( K_d \) of wild type BLIP and 23 alanine mutants for the interaction with TEM-1 and SME-1 were determined using a kinetic assay of β-lactamase inhibition. Several previous studies have focused on the TEM-1-BLIP complex (14, 15, 17, 18, 21–23). The use of SME-1 in this study allowed an additional investigation into the determinants of binding specificity. It was found that the specificity of binding is significantly altered by mutation of two charged residues, Glu\(^{73}\) and Lys\(^{74}\), that are buried in the structure of the TEM-1-BLIP complex as well as by residues located on two loops that insert into the active site pocket. This information was used to engineer a BLIP molecule with a 220,000-fold change in binding specificity relative to wild type.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes were purchased from New England Biolabs except for *Pfu* polymerase, which was purchased from Stratagene. Oligonucleotide primers were purchased from Integrated DNA Technologies. Talon cobalt resin was purchased from Clontech. Cephalosporin C was purchased from Sigma. Cation exchange columns (SP Fast Flow) were purchased from Amersham Biosciences.

**BLIP Cloning and PCR Mutagenesis**—Wild type, D49A, and F142A BLIPs with N-terminal His tags were constructed previously (15). All other BLIP mutants were constructed by overlapping PCR (24) and cloned into pGEX32 with an N-terminal His tag as previously described (15). The external PCR primers used to amplify BLIP were as follows: PD-bla1 (top strand, N-terminal), 5'-CGGGAGTCTGATTCTTA-GACGTCAGGGCCG-3'; MALBLI-2 (bottom strand C-terminal), 5'-GG-CGAAATCTAGATTAACAGGTCCTACAGGCC-3'. A SacI site in PD-bla1 and an XbaI site in MALBLI-2 allowed the PCR product to be digested with SacI and XbaI and cloned into SacI and XbaI-digested pTIP123 following treatment of the vector with calf intestinal alkaline phosphatase (15). The internal top and bottom primers used for constructing the BLIP mutants are shown in Table I. The double mutant E73A/Y50A was constructed by overlap extension PCR using the BLIP Y50A mutant as the template and E73A top and bottom as inside primers. The coding sequences of the R160A and W162A mutants were expressed and purified using the protocol published previously with minor changes (15). The plasmids containing either wild type BLIP or the mutants were electrotrophased into *E. coli* RB791 (equivalent to strain W3110 lacI\(^Q\)lacI\(^Q\)) (26). An overnight culture of each mutant was grown at 37 °C with shaking in 10 ml of LB medium in the presence of 12.5 μg/ml chloramphenicol. 1.5 liters of LB medium containing 12.5 μg/ml chloramphenicol were then inoculated using the 10 ml overnight culture. The culture was then grown at 37 °C until A\(_{500}\) = 1.2. For induction of BLIP, 3 μl isopropyl-\(\beta\)-D-thiogalactopyranoside was added to each culture, and the cultures were allowed to grow overnight at 25 °C. The cells were pelleted and frozen at −80 °C for at least 1 h. The frozen cells were then thawed and resuspended in 40 ml of B-Per (Pierce). The cell debris was removed by centrifugation, and the soluble protein in the supernatant was purified over a 1 ml TALON cobalt resin column (Clontech) according to the manufacturer’s instructions. A 10 mM imidazole wash step was utilized to remove protein from the column that bound less tightly than the His-tagged BLIP. BLIP was eluted using a buffer consisting of 50 mM imidazole in washing buffer (20 mM Tris-HCl and 500 mM NaCl, pH 8.0).
TEM-1 and SME-1 \(\beta\)-Lactamase Expression and Purification—

TEM-1 \(\beta\)-lactamase was purified to greater than 95% homogeneity as previously described (28). SME-1 was expressed and purified from \(E.\) coli as previously described (29).

Kinetic Inhibition Assay—The kinetic assay was performed as described previously with minor changes (15). The assays were performed in a 96-well quartz plate. 12 reactions were monitored simultaneously in a Tecan ultraviolet spectrophotometer controlled by Magellan (Phenix) software. 2 nM TEM-1 \(\beta\)-lactamase or 4 nM SME-1 \(\beta\)-lactamase was incubated with 12 different concentrations of BLIP for 1 h at 25 °C in 50 mM phosphate buffer (pH 7.0) containing 1 mg/ml bovine serum albumin. 100 \(\mu\)M cephaloridine \(C\) \(K_0\) is 700 \(\mu\)M for TEM-1 and 1300 \(\mu\)M for SME-1 was added to the \(\beta\)-lactamase/BLIP incubation buffer with a 12-channel pipetter. The final reaction volume was 0.3 ml, and hydrolysis of cephaloridine \(C\) was monitored at \(A_{\text{max}}\) (Fig. 1). Plots of the concentration of free \(\beta\)-lactamase versus inhibitor concentration were fit by nonlinear regression analysis to Equation 1,

\[
E_{\text{red}} = \frac{[E_0] + K_i \cdot \sqrt{[E_0] + [I_0] + K_i} + \left(4[E_0][I_0]\right)}{2}
\]

(Eq. 1)

where \([E_{\text{red}}]\) is the concentration of active \(\beta\)-lactamase calculated from the measured velocity and the activity and concentration of uninhibited \(\beta\)-lactamase, \([E_0]\) is the total \(\beta\)-lactamase concentration, and \([I_0]\) is the total inhibitor concentration (30). From the equation, apparent equilibrium dissociation constants \(K_i\) were determined.

RESULTS

Determination of the Functional Epitope for BLIP Binding to TEM-1 \(\beta\)-Lactamase—Alanine-scanning mutagenesis of BLIP was performed to identify the determinants critical for binding TEM-1 \(\beta\)-lactamase. Amino acid residues in BLIP within 4 Å from TEM-1 in the co-crystal structure were chosen for mutagenesis (17). Wild type BLIP and the 29 BLIP mutants were expressed in \(E.\) coli and purified (Fig. 1), and apparent binding constant data were obtained using a kinetic assay of inhibition of \(\beta\)-lactam hydrolysis (Fig. 2, Table II). Residues that result in a >10-fold decrease in binding affinity when mutated are considered components of the functional epitope. By this definition, 11 residues including Phe\(^{121}\), His\(^{146}\), Asp\(^{49}\), Tyr\(^{53}\), Lys\(^{74}\), Trp\(^{112}\), Phe\(^{142}\), His\(^{146}\), Trp\(^{156}\), Arg\(^{160}\), and Trp\(^{162}\) (Fig. 3) comprise the functional epitope. The critical residues are largely located in two patches near the center of the interface as well as in the two loops that insert in the active site pocket of the enzyme (Fig. 3).

As described above, two loops on BLIP (loop 1, residues 46–51; loop 2, residues 136–144) insert into the active site of TEM-1 \(\beta\)-lactamase (17). Within loop 1, Asp\(^{49}\) forms two salt bridges and two hydrogen bonds with four conserved TEM-1 residues that are essential for enzyme activity: Ser\(^{130}\), Lys\(^{234}\), Ser\(^{235}\), and Arg\(^{244}\) (17). The carboxylate of Asp\(^{49}\) assumes the position of the carboxylate of penicillin \(G\) in the acyl-enzyme complex of TEM-1 with penicillin \(G\) (17). Phe\(^{142}\) accounts for most of the van der Waals interactions in loop 2, and it mimics the benzyl group of penicillin \(G\) for binding TEM-1 (17). As expected based on these interactions and consistent with previous results, mutation of these residues to alanine results in >20-fold loss of binding affinity for TEM-1 \(\beta\)-lactamase (Table II). Other loop residues do not play a significant role in binding with the exception of Tyr\(^{50}\). Surprisingly, mutation of this residue to alanine results in a >50-fold increase in binding affinity. Tyr\(^{26}\) forms extensive van der Waals interactions with Pro\(^{107}\), Val\(^{116}\), and Met\(^{129}\) of TEM-1. In addition, Tyr\(^{26}\) forms an aromatic patch on BLIP together with residues Tyr\(^{51}\), Phe\(^{36}\), His\(^{41}\), and Tyr\(^{53}\). The aromatic ring of Tyr\(^{50}\) is located between the hydrophobic rings of Pro\(^{107}\) of TEM-1 and Tyr\(^{51}\) of BLIP. Perhaps the Tyr\(^{50}\) side chain hinders Asp\(^{49}\) or other residues in loop 1 from assuming optimal interactions in the active site of TEM-1 \(\beta\)-lactamase. For example, removal of the bulky Tyr\(^{50}\) side chain could result in better contacts between BLIP residues His\(^{41}\) and Tyr\(^{51}\) and the TEM-1 enzyme.

The x-ray structure of the BLIP-TEM-1 complex reveals several ordered water molecules at the interface between BLIP and the Glu\(^{99}\)-His\(^{112}\) region of \(\beta\)-lactamase (17). The water molecules are clustered near polar residues such as several serines on the concave surface of BLIP. Mutation of Ser\(^{135}\), Ser\(^{249}\), Ser\(^{71}\), and Ser\(^{113}\) at the interface had no effect on binding (Table II). It has been proposed that the intervening waters at the BLIP-TEM-1 interface may form an adaptable interface that allows BLIP to bind several different \(\beta\)-lactamase sequences (17). These results suggest that the BLIP side of this interface is also adaptable in that the serine residues do not contribute to binding affinity.

Two negatively charged BLIP residues in the interface, Glu\(^{31}\) and Glu\(^{72}\), do not contribute to binding affinity (Table II). There is a salt bridge between Glu\(^{31}\) and Lys\(^{315}\) of TEM-1 that...
The charge, which will be discussed below. Lys74 is fully buried at the interface and makes a salt bridge with Glu104 from TEM-1 (17). Schreiber and colleagues (14, 18) have shown that electrostatic effects strongly influence the association rate. A similar explanation may apply to Arg160, which is near the periphery of the interaction surface. However, the aliphatic portion of the side chain also makes packing interactions with His148, Trp150, and Trp162 in BLIP that, as described below, form an aromatic patch on BLIP that is critical for binding affinity. The only positively charged residue that did not affect binding when mutated to alanine was His41, Tyr50, and Tyr53, whereas the patch from domain two includes residues His148, Trp150, and Trp162. The aromatic patch from domain one includes residues Phe36, His41, Tyr50, and Tyr53, whereas the patch from domain two includes residues His148, Trp150, and Trp162. The figure was prepared with PyMOL (W. L. DeLano; available on the World Wide Web at www.pymol.org).

is partially exposed to the solvent (17). The solvation effect may limit the contribution of Glu31 to binding affinity. Glu73 is fully buried inside the interface and makes hydrogen bonds with Glu104, Tyr150, and Ser106 of TEM-1. Removal of the Glu73 side chain, however, does not affect binding affinity to TEM-1 (Table II).

In contrast, mutation of several positively charged residues on the binding surface of BLIP significantly increased $K_i$ values (Table II). The mutated residues include Lys74, Arg160, His41, and His148. The significance of the histidine residues may arise from their ring structure rather than the partial positive charge, which will be discussed below. Lys74 is fully buried at the interface and makes a salt bridge with Glu104 from TEM-1 and neutralizes a negative region formed by Glu73 of BLIP and Glu104 of TEM-1 (17). Schreiber and colleagues (14, 18) have shown that electrostatic effects strongly influence the association rate and thereby the equilibrium constant for the BLIP-TEM-1 interaction. It was observed that mutations that reduce the negative charge on either molecule increase the association rate. Since removal of the Lys74 side chain increases the negative charge of BLIP, the mutation may act by slowing the association rate. A similar explanation may apply to Arg160, which is near the periphery of the interaction surface. However, the aliphatic portion of the side chain also makes packing interactions with His148, Trp150, and Trp162 in BLIP that, as described below, form an aromatic patch on BLIP that is critical for binding affinity. The only positively charged residue that did not affect binding when mutated to alanine was...
Fig. 4. Electrostatic surface representation of TEM-1 and SME-1 β-lactamase. Regions of negative charge are shown in red, and positive charge is shown in blue. The boxed region of each enzyme is bound by BLIP. The yellow circle denotes the active site pocket. The figure was generated using the GRASP program (38) and the PDB coordinates 1BTL (TEM-1) (39) and 1DY6 (SME-1) (12).

Arg^{144}. This residue is at the edge of loop 2 on the periphery of the binding surface and remains largely exposed on complex formation. The lack of effect is not surprising from the standpoint of its limited direct interactions with β-lactamase, but it might have been expected to decrease affinity based on the change in electrostatics (14, 18).

In contrast to the diverse effect of mutating hydrophilic residues, the majority of the hydrophobic residues tested contribute significantly to the BLIP-TEM-1 interaction (Table II). The mutation of these residues to alanine increases $K_I$ values at least 20-fold. It should be noted that all hydrophobic residues mutated in this study are also aromatic residues. Two patches of aromatic residues account for most of the binding affinity, with His^{148}, Trp^{150}, and Trp^{160} forming one patch and Phe^{36}, His^{41}, Tyr^{47}, and Tyr^{53} near loop 1 forming the other (Fig. 3). Mutation of Trp^{150} results in the largest increase (370-fold) in the $K_I$ value among all mutants tested (Table II). The patch centered at His^{148} makes extensive interactions with Gln^{99}-Asn^{100} and Leu^{102}-Val^{103} in TEM-1 β-lactamase, whereas the patch near loop 1 makes contacts with Ser^{106}, Pro^{107}, Val^{108} of TEM-1. Leu^{102}, Val^{103} and Pro^{107}, Val^{108} are conserved positions in the loop-helix domain among several class A β-lactamases (10). In addition, a previous study from this laboratory demonstrated that residues Leu^{192}, Val^{193}, Ser^{196}, Pro^{197}, and Val^{208} in TEM-1 are important for binding to BLIP (23). Therefore, the residues in the critical aromatic patches in BLIP largely interact with TEM-1 residues that are also important for binding. This suggests that the residues in the aromatic patches make precise interactions with the TEM-1 residues in these regions.

**Determination of the Functional Epitope for BLIP Binding to SME-1 β-Lactamase.—**SME-1 β-lactamase is 33% identical to TEM-1 but differs significantly from TEM-1 in both surface charge and shape in the loop-helix region that BLIP binds (Fig. 4). The inhibition assay indicated that wild type BLIP inhibits SME-1 with a $K_I$ of 2.4 nM, which is ~5-fold weaker than the interaction with TEM-1 (Fig. 2, Table II). Clearly, the difference in both surface charge and shape of SME-1 does not abolish the protein-protein interaction. The differences between the TEM-1 and SME-1 β-lactamases provide an interesting system with which to investigate the determinants of binding specificity for BLIP.

The functional epitope of BLIP for binding SME-1 (>10-fold increase in $K_I$) consists of 12 residues: Phe^{36}, His^{41}, Arg^{44}, Tyr^{47}, Tyr^{50}, Glu^{73}, Trp^{112}, His^{148}, Trp^{150}, Arg^{160}, and Trp^{162} (Table II). The sequence requirements for the SME-1-BLIP interaction are, in general, similar to those for the TEM-1-BLIP interaction. For example, the two aromatic patches critical for the TEM-1-BLIP interaction are also critical for the SME-1-BLIP interaction. The loop 1 residue Asp^{90} that makes multiple interactions in the active site of TEM-1 is also important for binding to SME-1, as is the positively charged residue Arg^{160}. Finally, the multiple serine residues are not essential for the interaction of BLIP with either enzyme.

There are, however, two significant differences between the functional epitopes of BLIP for TEM-1 and SME-1. First, two charged residues buried at the interface strongly influence binding specificity. Glu^{73} is very important for binding to SME-1 β-lactamase but does not contribute to binding TEM-1 β-lactamase. In contrast, Lys^{74} is important for binding TEM-1, but the K74A substitution actually binds SME-1 10-fold tighter than wild type BLIP. As stated above, Lys^{74} of BLIP interacts with Glu^{104} of BLIP and Glu^{104} of TEM-1 β-lactamase. Based on a sequence alignment between TEM-1 and SME-1, the equivalent residue to Glu^{104} of TEM-1 is Tyr^{104} of SME-1, which would interact differently with Lys^{74}. In addition, the topology of the loop-helix region is somewhat different between TEM-1 and SME-1 in this region (Fig. 4), which could alter multiple interactions. Finally, the mutation of Lys^{74} increases the negative charge on BLIP, which may augment electrostatic interactions with the positively charged SME-1 (14). It should be noted, however, that mutation of Arg^{144} and Arg^{160} would have a similar effect on electrostatics, but these mutations either do not affect or are detrimental for binding to SME-1 (Table II).

The second significant difference in sequence requirements for binding the enzymes is that mutation of loop 1 residues Tyr^{50} and Tyr^{51} either improves or does not affect binding of BLIP to TEM-1 β-lactamase but significantly reduces affinity for SME-1. In contrast, mutation of loop 2 residue Phe^{142} significantly reduces binding to TEM-1 but has a relatively minor effect on binding affinity for SME-1. In fact, none of the loop 2 residues examined displayed significantly altered $K_I$ values for SME-1 when mutated to alanine. Taken together, these data suggest that loop 1 of BLIP does not make optimal interactions with the TEM-1 active site, whereas loop 2 of BLIP does not interact strongly with the SME-1 active site. Subtle differences in the active sites of the enzymes may influence the loop interactions. Alternatively, differences in the interactions of BLIP with the loop-helix region of the β-lactamases may alter the positioning of the loops with respect to the enzymes. Although the exact mechanisms for changes in binding affinity are not clear, it is apparent that a relatively small number of residues, Glu^{73}, Lys^{74}, Tyr^{50}, Tyr^{51}, and Phe^{142}, are important determinants of the binding specificity of BLIP.

**Engineering the Binding Specificity of BLIP.—**The observation that single alanine substitutions result in large changes in the binding specificity of BLIP suggested that the binding specificity could be drastically altered with just a few changes in sequence (Fig. 5). For example, the BLIP mutant containing the Y50A substitution binds TEM-1 β-lactamase 50-fold tighter than wild type but binds SME-1 13-fold weaker. In addition, the E73A mutant binds TEM-1 with the same affinity as wild type BLIP, but binds SME-1 18,300-fold weaker than wild type (Fig. 5, Table II). Because the Tyr^{50} and Glu^{73} residues are not in direct contact in the BLIP structure and mutations of residues that are not in contact often act additively when combined (31), it was reasoned that the Y50A/E73A double mutant would possess the combined effects of the mutations and thereby exhibit a vast difference in binding specificity for TEM-1 versus SME-1 β-lactamase. This was found to be the case, since the Y50A/E73A double mutant binds TEM-1 β-lactamase ~10-fold tighter than wild type BLIP, whereas it binds SME-1 18,300-fold weaker than wild type (Fig. 5, Table II). As a result, wild type BLIP exhibits a 5-fold preference for binding TEM-1 versus SME-1 β-lactamase, whereas the double mutant displays a 1.1 million-fold preference for binding TEM-1 versus SME-1.


β-lactamase (Fig. 5, Table II). Therefore, the binding specificity of BLIP can be drastically altered with relatively few amino acid substitutions.

DISCUSSION

The common functional epitope of BLIP for binding both TEM-1 and SME-1 β-lactamase consists of 9 of the 23 residues mutagenized and includes positions Phe36, His41, Asp49, Tyr53, Trp112, His148, Trp150, Arg160, and Trp162 (Fig. 3). These residues represent two aromatic patches in addition to Asp49 from loop 1. It is interesting that each domain of BLIP contributes an aromatic patch (Fig. 3). The locations of the patches with respect to the binding surfaces, however, are somewhat different. For example, the patch from domain one includes residues Phe36, His41, and Tyr53 and is located in the center of the binding interface provided by domain one. The periphery of this patch contains the polar residues Ser35, Ser39, and Ser71, which do not contribute substantially to binding of either β-lactamase. This type of organization has been noted for several protein-protein interaction interfaces, and it has been proposed that the major role of the residues on the periphery of the critical residues is to serve as an “O-ring” to occlude bulk solvent from the hotspot (8).

The aromatic patch from domain two includes residues His148, Trp150, and Trp162. Although not an aromatic residue, Arg160 is critical for binding and also packs into this hotspot (Fig. 3). In contrast to the patch from domain one, this patch is located near the periphery of the binding interface (Fig. 3). For example, His148, Trp150, Arg160, and Trp162 retain 1.1, 28.6, 71.2, and 26.0 Å² of accessible surface area in the complex, respectively, due to their position near the edge of the interface. In contrast, Phe36, His41, and Tyr53 from patch one exhibit only 0.3, 0.0, and 0.46 Å² of accessible surface area in the complex, respectively, due to their more centered position in the domain one binding interface. Therefore, it appears that the aromatic patch in domain two does not require a ring of surrounding residues to protect the hotspot (8). Finally, it is of interest that Trp112 is critical for binding but does not belong to either of the aromatic patches. Rather, Trp112 serves as a bridge between the aromatic patches from domains one and two (Fig. 3B).

Analysis of an extensive alanine-scanning database has shown that the amino acid composition of hotspots is enriched for tryptophan, arginine, tyrosine, aspartate, isoleucine, and histidine residues (8, 32). With the exception of isoleucine, these residues are also enriched on the binding surface of BLIP (17). Of the 23 BLIP residues within 4 Å of the TEM-1 β-lactamase surface, 13 are within this class. In general, these residues were also found to be important for the β-lactamase-β-lactamase interaction (Table II). For example, all three of the tryptophan residues, both of the histidine residues, and the loop 1 aspartate residue at the interface are important for binding to both TEM-1 and SME-1 β-lactamase. In contrast, only one of the four tyrosine residues at the interface contributes significantly to binding TEM-1 β-lactamase based on the mutation results. However, two additional tyrosines on loop 1, Tyr50 and Tyr51, are important for binding SME-1 β-lactamase.

Phenylalanine is not enriched at hotspots in the alanine-scanning database (32). Nevertheless, it is important at the BLIP interface in that Phe36 is a critical residue from one of the aromatic patches and Phe142 on loop 2 is critical for binding TEM-1 β-lactamase. These results are consistent with an analysis of structurally conserved residues and binding energy hotspots that showed tryptophan and phenylalanine are conserved at interaction surfaces but not on the exposed surface of proteins (33). In addition, analysis of the alanine-scanning database as well as structurally conserved residues in binding sites indicates that serine residues are very rarely found in energy hotspots (8, 33). This is true for the BLIP interface as well in that none of the four serines at the interaction interface make a significant contribution to the binding of either β-lactamase. A similar result was reported for the peripheral polar residues in the human growth hormone and hormone receptor complex (20). The serines on the periphery of BLIP interact with water molecules that bridge the interface with β-lactamase. The intervening water molecules may contribute to the adaptability of the periphery of the interface.

BLIP binds a number of β-lactamases from both Gram-negative and Gram-positive bacteria (16). Examination of the binding of the alanine scan mutants to TEM-1 and SME-1 β-lactamase indicates that most of the positions, including the two aromatic patches, make similar contributions to binding each enzyme. Only a relatively small number of residues influence binding specificity. Two fully buried charged residues in BLIP, Glu73 and Lys74, contribute to the specificity of binding, as do Tyr50 and Tyr51 located on loop 1 and Phe142 on loop 2. Schreiber and colleagues (14, 18) have shown that alteration of charged residues on the periphery of the BLIP-TEM-1 β-lactamase interface can strongly influence the association rate and thereby the equilibrium binding constant. Although Glu73 and Lys74 are not in the periphery but rather fully buried in the binding interface, it is possible that they could act on the association rate. It has previously been suggested that buried electrostatic interactions may contribute to specificity (34). Further studies are required, however, to determine the mechanism for the specificity change associated with Glu73 and Lys74.

The roles of Tyr50, Tyr51, and Phe142 in binding specificity may be due to altered interactions between loop 1 and loop 2 with the TEM-1 and SME-1 active sites. Removal of the Tyr50 side chain significantly increases binding affinity of BLIP with TEM-1 but not SME-1 β-lactamase (Table II). The side chain of Tyr50 fits into a pocket formed by TEM-1 residues Pro107, Met129, and Val216 (17). Although the catalytic machinery of the SME-1 active site is similar to TEM-1, there are several differences that could prevent similar interactions from occurring. For example, the equivalent Tyr52-interacting residues in SME-1 are Pro107, Tyr129, and Thr216 that form a somewhat
Altered binding pocket. In contrast, Tyr^{51} packs onto Tyr^{50} but does not directly contact β-lactamase. Changes at Tyr^{51}, however, could alter the position of Tyr^{50} or the conformation of loop 1 and thereby affect binding.

Replacement of the Phe^{142} side chain with alanine significantly reduces binding affinity of BLIP with TEM-1 but not SME-1 β-lactamase. The Phe^{142} side chain interacts with several TEM-1 active site residues including Glu^{104}, Tyr^{105}, Asn^{170}, Glu^{240}. The equivalent residues in SME-1 are Tyr^{104}, His^{105}, Asn^{170}, Cys^{238}, and Ala^{240}. These differences may result in a poor interaction between loop 2 and SME-1 and therefore little contribution from Phe^{142}.

The ability to engineer protein-protein interactions or design binding sites could facilitate the design of new therapeutics. One interesting challenge is to engineer the specificity of protein interactions. Wild type BLIP exhibits a 5-fold binding affinity preference for TEM-1 versus SME-1 β-lactamase. The results of the mutational analysis, however, suggested that the binding specificity of BLIP could be tailored to interact strongly with one substrate but not another. For example, the K74A mutant exhibits a significant change in binding specificity in that it binds SME-1 460-fold tighter than TEM-1 rather than 5-fold weaker as seen with wild type BLIP. Thus, a single amino acid substitution in BLIP resulted in a 2300-fold change in specificity versus wild type.

The observation that the E73A mutation greatly reduced BLIP binding to SME-1 while not affecting binding to TEM-1 as well as the finding that the Y50A mutant bound more tightly to TEM-1 but not SME-1 suggested that the Y50A/E73A double mutant would exhibit greatly altered specificity. This was the case, since the double mutant exhibited a 1.1 million-fold preference for binding TEM-1 versus SME-1 β-lactamase. Because wild type BLIP exhibits a 5-fold preference for TEM-1 the double mutant exhibits a 220,000-fold change in binding specificity.

One reason for the changes in specificity observed here is that the Tyr^{50} side chain not only does not favor binding to TEM-1 β-lactamase; it actually decreases affinity by an order of magnitude. Similarly, Lys^{74} side chain decreases affinity of BLIP for SME-1 by more than an order of magnitude. A large increase in affinity upon mutation of a residue has not been frequently observed in other protein-protein interfaces (6, 7, 35, 36). Nevertheless, it is clear from these results that the binding affinity of BLIP for either TEM-1 or SME-1 β-lactamase has not been optimized by evolution. This is not surprising in that it is very unlikely that BLIP evolved to bind either TEM-1 or SME-1 β-lactamase, since BLIP is produced by a Gram-positive soil bacterium, and TEM-1 and SME-1 β-lactamase are found in Gram-negative enteric bacteria (37). The role of BLIP in the biology of S. clavuligerus is not known, so it is difficult to speculate on the selective pressures that did shape BLIP function.

Although BLIP binds to several class A β-lactamases, it does not efficiently bind other classes of β-lactamase or penicillin-binding proteins (16). The results presented here suggest that BLIP can be engineered to serve as an inhibitor of a number of clinically important β-lactamases and penicillin-binding proteins. More generally, the results suggest that the specificity of protein-protein interactions can be modified with limited amino acid substitutions.

Acknowledgments—We thank Hiram Gilbert for assistance with the β-lactamase inhibition assay, Fahd Majdudin for providing SME-1 β-lactamase, and Wanzhi Huang for providing technical assistance. We also thank Natalie Strynadka for the coordinates of the BLIP/TEM-1 β-lactamase structure.

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J. Biol. Chem. 2003, 278:45706-45712.
doi: 10.1074/jbc.M308572200 originally published online August 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308572200

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