Dissecting the Protein-Protein Interface between \( \beta \)-Lactamase Inhibitory Protein and Class A \( \beta \)-Lactamases*  

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\( \beta \)-Lactamase inhibitory protein (BLIP) binds and inhibits a diverse collection of class A \( \beta \)-lactamases at a wide range of affinities. Alanine-scanning mutagenesis was previously performed to identify the amino acid sequence requirements of BLIP for inhibiting TEM-1 \( \beta \)-lactamase and SME-1 \( \beta \)-lactamase. Two hotspots of binding energy, one from each domain of BLIP, were identified (Zhang, Z., and Palzkill, T. (2003) J. Biol. Chem. 278, 45706–45712). This study has been extended to examine the amino acid sequence requirements of BLIP for binding to the SHV-1 \( \beta \)-lactamase, which is a poor binding substrate (K\text{I} = 1.1 \mu M), and the Bacillus anthracis Bla1 enzyme (K\text{I} = 2.5 \mu M). The two hotspots previously identified as important for binding TEM-1 and SME-1 \( \beta \)-lactamase were also found to be important for binding Bla1. The hotspot from the second domain of BLIP, however, does not make substantial contributions to SHV-1 binding. This may explain why BLIP binds to SHV-1 \( \beta \)-lactamase with much weaker affinity than to the other three enzymes. Three regions, including two loops that insert into the active pocket of TEM-1 \( \beta \)-lactamase and the Glu-73—Lys-74 buried charge motif, exhibit strikingly different effects on the binding affinity of BLIP toward the various enzymes when mutated and, therefore, act as specificity determinants. Analysis of double mutants of BLIP that combine specificity-determining residues suggests that these residues contribute to the poor affinity between the second domain of BLIP and SHV-1 \( \beta \)-lactamase.

Protein-protein interactions play a critical role in most cellular processes. An understanding of the molecular basis of these interactions is required to engineer novel protein-protein interactions or to design drugs that manipulate these interactions in a rational way (2). Protein binding surfaces exhibit specific chemical and geometric characteristics that determine affinity and specificity for binding to their targets (2–7). It is difficult to design small molecules that mimic the interactions of a protein interface because of the generally very large binding area and the large number of residues participating in the interface. Alanine-scanning mutagenesis, however, indicates that only a small number of residues on the interface make significant energetic contributions to binding, and these residues are defined as a hot spot or functional epitope (2, 8–10).

The existence of hot spots makes the task of designing small molecules that alter protein-protein interactions less daunting. Several attempts have been made to understand the molecular basis of protein-protein interactions by systematic analysis of physiochemical properties or alanine-scanning mutagenesis data on protein-protein interfaces (5, 7, 11, 12). Some general rules have been proposed, but they seldom hold true for all protein-protein interfaces. Conserved polar residues have been proposed to constitute hot spots because they are frequently found in interfaces (13). Hydrophobic residues or large aromatic residues are also frequently found in hot spots, and interfaces usually bury a large hydrophobic surface area (14). Neither polar interactions nor hydrophobic interactions alone, however, are sufficient to identify binding sites (15, 16). The factors that differentiate residues in a hot spot from other residues on the interface or other regions of the protein surface remain elusive. Therefore, additional structure-function studies are required to understand the key components of protein-protein interactions.

\( \beta \)-Lactam antibiotics, such as the penicillins and cephalosporins, are commonly prescribed anti-microbial drugs. The extensive use of these drugs has resulted in widespread \( \beta \)-lactam antibiotic resistance, which is becoming a threat to public health. The expression of \( \beta \)-lactamases that catalyze the hydrolysis of \( \beta \)-lactams is a common mechanism for bacterial resistance to these drugs. \( \beta \)-Lactamases have been grouped into four classes according to primary sequence alignments (17). *Escherichia coli* TEM-1, *Serratia marcescens* SME-1, Klebsiella pneumoniae SHV-1, and Bacillus anthracis Bla1 are all class A \( \beta \)-lactamases that share at least 30% sequence identity (Table I). The x-ray crystal structures of the TEM-1, SME-1, and SHV-1 \( \beta \)-lactamases indicate that they possess a similar-fold (18–20). The three-dimensional structure of Bla1 is not yet available. All four of these enzymes are able to hydrolyze most penicillins and early generation cephalosporins but are unable to efficiently hydrolyze third generation cephalosporins. The SME-1 \( \beta \)-lactamase is also capable of hydrolyzing carbapenem antibiotics (21).

The widespread antibiotic resistance due to \( \beta \)-lactamase production has resulted in a need to develop novel inhibitors of these enzymes to aid the treatment of infection. \( \beta \)-Lactamase inhibitory protein (BLIP) is a 17-kDa protein produced by *Streptomyces clavuligerus* that inhibits class A \( \beta \)-lactamases with a wide range of affinities (Table I) (22, 23). BLIP consists of two tandemly linked 76-amino acid domains. The co-crystal structure of the TEM-1 \( \beta \)-lactamase-BLIP complex indicates

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‡ The abbreviations used are: BLIP, \( \beta \)-lactamase inhibitory protein; PDB, Protein Data Bank.
that BLIP uses its 7-strand β-sheet to clamp over the loop-helix region of TEM-1 (residues 99–114) (Fig. 1) (23). Sequence alignments of class A β-lactamases that are inhibited by BLIP do not exhibit extensive amino acid sequence conservation in this region (23). In addition, two loops in BLIP, one from each domain, insert into the active site of TEM-1 β-lactamase to block the binding of substrates. The interaction area between BLIP and β-lactamase (2636 Å²) is one of the largest among known protein-protein interactions (23). Several previous studies have focused on the TEM-1-BLIP complex (1, 23–29). We previously employed alanine-scanning mutagenesis to determine the functional epitopes of BLIP for binding the TEM-1 and SME-1 β-lactamases (1). Two patches of residues (patch 1, residues Phe-36, His-41, and Tyr-53; patch 2, residues His-148, Trp-150, Arg-160, and Trp-162), one from each domain of BLIP, were shown to dominate the binding affinity. In addition, the two loops that insert into the active pocket of TEM-1 as well as a Glu-73-Lys-74 motif that is fully buried in the interface are important determinants of the specificity of BLIP binding to TEM-1 versus SME-1 β-lactamase (1).

In this study the alanine-scanning mutagenesis experiments were continued to determine the functional epitope of BLIP for binding two additional class A β-lactamases, SHV-1 and Bla1. BLIP binds to SHV-1 \(K_i = 1.1 \text{ μM}\) much weaker than it does to TEM-1 \(K_i = 0.5 \text{ μM}\) even though the enzymes share 68% sequence identity and a similar substrate spectrum (18, 25). In contrast, BLIP inhibits the SME-1 and Bla1 β-lactamases efficiently \(K_i = 2.5 \text{ μM}\) despite the fact that SME-1 and Bla1 share only about 30% identity to TEM-1 β-lactamase. Examination of BLIP binding to the SHV-1 and Bla1 β-lactamases provides an interesting comparison with the previous study on binding of BLIP to the TEM-1 and SME-1 enzymes (1).

The results suggest that the weak interaction between SHV-1 β-lactamase and BLIP is due to a lack of contribution of residues from the second domain of BLIP.

**EXPERIMENTAL PROCEDURES**

**Materials—**All enzymes were purchased from New England Biolabs except the 7F11 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. Nitrocefin was purchased from BD Biosciences. Cation exchange columns (SP Fast Flow) were purchased from Amersham Biosciences.

**BLIP Mutagenesis, Cloning, and Purification—**All single mutants of BLIP were constructed previously (1, 25). The construction and cloning of double mutants of BLIP follow the same protocol using overlapping PCR (30). The DNA sequence of each mutant was confirmed by the dyeoxy chain termination method using an ABI 3100 capillary DNA sequencer. The purification of BLIP mutants followed the procedure described previously (1). The purity of the BLIP samples was determined by SDS-PAGE. BLIP concentrations were determined using a Bradford assay with a standard curve calibrated by quantitative amino acid analysis (31).

**Sequence Alignment of Class A β-Lactamases—**Sequences of class A β-lactamases were retrieved from the National Center for Biotechnology Information protein data base. The sequence alignment was done using ClustalW WWW Service at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw) (32).

**Molecular Modeling—**Bla1 was modeled using SwissModel first approach mode at www.expasy.org/swissmod (33–35). To prevent bias, the modeling was carried out three times with different choices of templates. In trial 1, uncomplexed TEM-1 (1BTL) and SHV-1 (1ISH) were retrieved from the PDB. Both TEM-1 and SHV-1 are typical class A β-lactamases. The PDB files were then edited according to the requirements of the SwissModel server. The edited PDB files were then input as templates for the modeling. In trial 2, chain A of the TEM1-BLIP complex (1JTG), PC1 (3BLM), SME-1 (1D6Y), and SHV-1 (1ISH) were retrieved and edited as templates for the modeling. PC1 and SME-1 β-lactamases were used to increase diversity because of their unusual structures compared with other class A β-lactamases. In trial 3, the server was allowed to search for templates through the ExNR3-3D data base, which is a sequence data base designed for the purpose of finding templates for SwissModeling. The server found 87 available structures with more than 24% identity and used them as templates for the modeling. This setting included more diversified templates. All three methods gave basically the same Bla1 structure in the area of interest (the root mean square deviation among loop-helix domains of all three modeled Bla1 is ~0.5 Å). All PDB files were superimposed to 1JTG using Swiss PdbViewer before submission in setting 1 and 2. The returned output was a final model of Bla1 together with the superimposed templates as a Swiss-PdbViewer project file. The superimposed model structure was used as a reference to facilitate interpretation of the binding results.

**β-Lactamase Inhibition Assay—**BLIP binding was assessed using an inhibition assay as described previously (1). The assays were performed in a 96-well quartz plate. 12 reactions were monitored simultaneously in a Tecan ultraviolet spectrophotometer controlled by Magellan (Phe-9252) software. 5 nM SHV-1 β-lactamase or 1 nM Bla1 β-lactamase was incubated with 12 varying concentrations of BLIP for at least 1 h at 25 °C in 50 mM phosphate buffer (pH 7.0) containing 1 mg/ml bovine serum albumin. 50 μM cephalosporin C \(K_i = 250 \text{ μM}\) for SHV-1 was added to the SHV-1/BLIP incubation buffer, whereas 10 μM nitrocefin \(K_m = 19 \text{ μM}\) for Bla1 (36) was added to the Bla1/BLIP incubation buffer with a 12-channel pipetter. The final reaction volume was 0.3 ml. Hydrolysis of cephalosporin C was monitored at A570m, whereas hydrolysis of nitrocefin was monitored at A480. Plots of the concentration of free β-lactamase versus inhibitor concentration were fit by nonlinear regression analysis to Equation 1,

\[
E_{\text{free}} = [E]_0 + [I] + K_i - \sqrt{[E]_0 + [I] + K_i^2} - (4[E]_0[I]_0)
\]

(Eq. 1)
where $[E_{\text{rest}}]$ is the concentration of active β-lactamase calculated from the measured velocity and the activity and concentration of uninhibited β-lactamase, $[E_i]$ is the total β-lactamase concentration, and $[I_j]$ is the total inhibitor concentration (37). The apparent equilibrium dissociation constants $K_i$ were determined from the fit of the data to the equation.

RESULTS AND DISCUSSION

**Determination of the Functional Epitope for BLIP Binding to SHV-1 β-Lactamase**—SHV-1 is a class A β-lactamase that is most commonly found in the Gram-negative bacterium *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (38). SHV-1 β-lactamase shares 68% amino acid sequence identity with TEM-1 and exhibits a very similar β-lactam substrate spectrum. The three-dimensional structures of the enzymes are superimposable with the α-carbon atom overlay within a root mean square deviation of 1.4 Å (18). SHV-1 β-lactamase, however, is significantly different from TEM-1 with respect to binding to BLIP. BLIP binds and inhibits TEM-1 β-lactamase at high affinity ($K_i = 0.5$ nm), whereas it inhibits SHV-1 weakly ($K_i = 1.1$ μm) (25). It is, therefore, an interesting model to gain insight into the determinants of affinity and thereby specificity for the binding of BLIP to class A β-lactamasases. For this purpose, 23 alanine mutants of BLIP that were constructed previously were tested for binding to SHV-1 β-lactamase. The 23 mutated residues were originally chosen because they are within 4 Å of TEM-1 β-lactamase in the TEM-1-BLIP complex structure (1, 23). The residues comprising the functional epitope of BLIP for binding to SHV-1 β-lactamase are defined as those that exhibit a greater than 10-fold increase in $K_i$ value when mutated to alanine compared with wild type BLIP. According to this definition, the functional epitope consists of 5 residues including Phe-36, His-41, Asp-49, Tyr-53, and Trp-150 (Table II, Figs. 2 and 3). Four of the critical residues are from the first domain of BLIP, whereas Trp-150 is the only critical residue from the second domain (Table II, Figs. 2 and 3).

| BLIP variant | $K_i$ for TEM-1 | $K_i$ for SME-1 | $K_i$ for SHV-1 | $K_i$ for Bla1 |
|--------------|----------------|----------------|----------------|----------------|
| Wild type    | 0.5 ± 0.1*     | 2.4 ± 0.2*     | 1,130 ± 10     | 2.5 ± 0.9      |
| E31A         | 2.0 ± 0.5*     | 7.7 ± 1.9*     | 3,385 ± 643    | 5.5 ± 2.1      |
| S25A         | 0.5 ± 0.2*     | 1.8 ± 0.6*     | 227 ± 35       | 0.5 ± 0.1      |
| F36A         | 40 ± 15*       | 120 ± 17*      | ~120,000       | 511 ± 147      |
| S39A         | 0.3 ± 0.1*     | 1.4 ± 0.1*     | 225 ± 16       | 0.2 ± 0.06     |
| H41A         | 34 ± 10*       | 189 ± 5*       | 20,500 ± 7,778 | 173 ± 27       |
| G48A         | 0.7 ± 0.2*     | 2.6 ± 1.2*     | 550 ± 0.3      | 1.3 ± 0.3      |
| D49A         | 20 ± 4*        | 185 ± 30*      | ~50,000        | 167 ± 20       |
| Y50A         | 0.011 ± 0.004* | 32 ± 10*       | ~34 ± 3        | 1.1 ± 0.4      |
| Y51A         | 0.5 ± 0.03*    | 76 ± 24*       | 391 ± 57       | 2.3 ± 0.9      |
| Y53A         | 21 ± 2*        | 223 ± 64*      | ~5,000         | 39 ± 9         |
| S71A         | 0.2 ± 0.06*    | 1.9 ± 0.6*     | 476 ± 63       | 0.5 ± 0.2      |
| E73A         | 0.4 ± 0.06*    | 2,700 ± 71*    | 40 ± 8         | 0.00 ± 0.04    |
| K74A         | 46 ± 8*        | 0.1 ± 0.01*    | 783 ± 46       | 1.1 ± 0.4      |
| W112A        | 13 ± 3*        | 2,600 ± 706*   | 5,700 ± 2,800  | 18 ± 7         |
| S113A        | 0.11 ± 0.006*  | 2.7 ± 0.6*     | 402 ± 90       | 0.7 ± 0.07     |
| G141A        | 1.8 ± 0.2*     | 3.1 ± 0.5*     | 562 ± 42       | 0.6 ± 0.3      |
| F142A        | 16 ± 3*        | 15 ± 6*        | 1,800 ± 360    | 4.3 ± 1.6      |
| Y143A        | 0.6 ± 0.2*     | 11 ± 1*        | 50 ± 15        | 0.5 ± 0.1      |
| R144A        | 0.6 ± 0.2*     | 1.7 ± 0.4*     | 634 ± 78       | 0.3 ± 0.1      |
| H148A        | 21 ± 2*        | 1,400 ± 300*   | 7,467 ± 2,631  | 110 ± 22       |
| W150A        | 184 ± 52*      | 830 ± 190*     | 23,000 ± 10,000| 624 ± 154      |
| R160A        | 11 ± 2*        | 322 ± 125*     | 3,500 ± 754    | 25 ± 2         |
| W162A        | 20 ± 3*        | 97 ± 19*       | 2,767 ± 666    | 55 ± 17        |
| E73A/Y50A    | 0.04 ± 0.01*   | 40,000 ± 1400* | 18 ± 1         | 1.7 ± 0.4      |
| Y50A/Y143A   | 0.07 ± 0.02    | 62 ± 9         | 34 ± 4         | 0.8 ± 0.05     |
| E73A/Y143A   | 2.5 ± 0.3      | ~50,000        | 51 ± 0.7       | 0.7 ± 0.1      |
| W112A/Y143A  | 12 ± 6         | 1,800 ± 400    | 566 ± 175      | 8.9 ± 3.0      |
| W150A/Y143A  | 784 ± 138      | 11,000 ± 500   | 6,000 ± 350    | 175 ± 58       |

* a Data were published previously (1).

where $K_m$ is the total β-lactamase concentration, and $V_{max}$ is the total inhibitor concentration (37). The apparent equilibrium dissociation constants $K_i$ were determined from the fit of the data to the equation.
collision, in turn, could also effect interactions between other BLIP residues and SHV-1 by altering distances between residues in the interface.

**Determination of the Functional Epitope for BLIP Binding to Bla1**—Bla1 is a class A β-lactamase encoded on the chromosome of the Gram-positive bacterium *B. anthracis* (39). It is of interest that BLIP inhibits Bla1 with a $K_i$ of 2.5 nm despite Bla1 having only ~30% sequence identity to TEM-1 β-lactamase (Table I). The set of 23 alanine mutants of BLIP was tested for its ability to inhibit Bla1 (Table II). Alanine substitutions at 8 positions resulted in a >10-fold increase in $K_i$, including Phe-36, His-41, Asp-49, Tyr-53, His-148, Trp-150, Arg-160, and Trp-162 (Table II, Fig. 3). With the exception of Asp-49, which is in a loop that inserts into the β-lactamase active site, these residues clustered in the two patches of residues that were previously identified as functional epitopes of BLIP for binding the TEM-1 and SME-1 enzymes (1). Therefore, the functional epitope of BLIP is similar for all of the high affinity interactions, whereas the epitope for the weak interaction with SHV-1 is much smaller and is a subset of the high affinity epitope.

**Specificity Determinants**—Specificity is an important component of protein-protein interactions. Thousands of proteins are crowded in a cell, carrying out functions that are dependent on specific binding events. Although a great deal is understood on the molecular basis of interactions, less is known about the determinants of specificity. BLIP interacts with several different class A β-lactamases with varying affinities and is, therefore, a good model protein with which to study specificity determinants of protein-protein interactions.

In our previous study of the determinants of binding of BLIP with the TEM-1 and SME-1 β-lactamases, three specificity-determining regions were identified in BLIP including loop 1, loop 2, and a Glu-73–Lys-74 motif that is buried in the binding interface (Fig. 2) (1). A specificity determinant is defined as a residue that, when mutated to alanine, has a significantly different effect on binding affinity for different targets. For example, the Y50A substitution in loop 1 increases binding affinity of BLIP for TEM-1 β-lactamase by 45-fold but decreases the affinity of BLIP for SME-1 β-lactamase by 15-fold (1). The data for binding of the 23 BLIP alanine-scanning mutants to the SHV-1 and Bla1 enzymes also support a role for loop 1, loop 2, and the Glu-73–Lys-74 motif as specificity determinants (Table II). Alanine substitutions at any of three positions including Tyr-50 in loop 1, Tyr-143 in loop 2, or Glu-73 in the Glu-73–Lys-74 motif as specificity determinants (Table II). The E73A mutant also binds to Bla1 40-fold tighter than wild type BLIP (Table II). Interestingly, all these residues contact a similar region in class A β-lactamases, which includes Glu-104–Tyr-105–Ser-106–Pro-107 in TEM-1, which are located at the tip of peptide substrates.

![Fig. 2. Functional epitopes of BLIP for binding four class A β-lactamases.](image-url)

![Fig. 3. Comparison of ΔΔG values for binding of the BLIP alanine mutants to TEM-1 (black bars), SME-1 (red bars), SHV-1 (green bars), and Bla1 (yellow bars).](image-url)
loop-helix region and at the mouth of active pocket (Fig. 1).

The interaction of the three specificity-determining regions with the four class A β-lactamases used in the study is shown in Fig. 4. Tyr-105 of TEM-1 β-lactamase rearranges its side chain into a crowded space surrounded by the three specificity-determining regions in BLIP to prevent collision with Tyr-50 of BLIP (Fig. 4A) (23). The side chain of Tyr-105 is within 4 Å distance from surrounding residues in TEM-1-BLIP complex structure. SHV-1 and Bla1 also have a tyrosine at the same position. All three enzymes exhibit a stronger interaction with Y50A BLIP than with wild type BLIP (Table II). In SME-1, a histidine residue occupies the corresponding position, which would be expected to alter the local interactions. The shorter side chain of histidine may avoid collision with Tyr-50, and the partially positive charge could form a cation–π interaction with Tyr-50 of BLIP, which could contribute to binding affinity and explain why the Y50A mutation decreases the binding affinity between SME-1 and BLIP by 13-fold.

The Glu-73–Lys-74 motif is a BLIP specificity determinant that is buried in the TEM-1/BLIP interface (23). Lys-74 is part of the functional epitope for binding to TEM-1 β-lactamase and forms a salt bridge with Glu-104 and a hydrogen bond with the main chain of Gly-141 of TEM-1 (23). Modeling suggests Tyr-104 in SME-1 collides with Lys-74 in the complex, which could explain why the BLIP K74A protein is a much stronger inhibitor than wild type BLIP for binding to SME-1 β-lactamase (1). SHV-1 and Bla1 have an aspartate at position 104, which may be too short to form a strong salt bridge with Lys-74. This could explain why the K74A substitution does not exhibit large differences in binding to SHV-1 and Bla1 compared with wild-type BLIP. Glu-73, which forms a hydrogen bond with the backbone of TEM-1, does not play a significant role in binding between TEM-1 and BLIP. However, the E73A mutant inhibits SHV-1 and Bla1 with much higher affinity than wild type BLIP (Table II). Both SHV-1 and Bla1 have an aspartate at position 104, which is different from Glu-104 in TEM-1 by missing only one CH₂ group. Modeling suggests the shorter side chain of Asp-104 in SHV-1 brings the negative carboxyl group to a distance of less than 3 Å to the negative side chain of Glu-73 provided that no conformational change occurs upon binding, whereas the side chain carboxyl group of Glu-104 of TEM-1 is further away (~6 Å) from that of Glu-73 of BLIP in TEM-1-BLIP complex (Fig. 4B). The E73A substitution would eliminate this adverse interaction and thereby increase binding affinity to SHV-1 and Bla1. It is also of interest that a role for the Glu-to-Asp difference at position 104 of SHV-1 β-lactamase in determining the affinity of BLIP has previously been suggested based on modeling of SHV-1 with BLIP (18).

Tyr-143 in loop 2 of BLIP is another specificity determinant for binding to class A β-lactamases in that the alanine substitution at this position does not alter binding to TEM-1 but greatly increases affinity for SHV-1 β-lactamase (Table II). Tyr-143 is in contact with Glu-104 in the TEM-1/BLIP structure (23). As stated above, SHV-1 contains an aspartate at position 104. The replacement of the Tyr-143 side chain with alanine could result in a more favorable interaction between Lys-74 of BLIP and Asp-104 of SHV-1. Note also that the Bla1 enzyme, which also has Asp-104, is bound 5-fold tighter by Tyr-143 mutant than wild-type BLIP, whereas the TEM-1 and SME-1 enzymes, which have a glutamate or tyrosine at this position, do not exhibit significant differences in binding to the Y143A mutant compared with wild type BLIP.

**Additivity Relationships between Alanine Substitutions**—Double mutants were constructed to determine the additivity relationships between BLIP residues that contribute to binding affinity. The specificity-determining residues Tyr-50, Glu-73, and Tyr-143 make contacts with a similar region in class A β-lactamases, and therefore, it was of interest to determine whether these residues act independently of one another. This was addressed by constructing the BLIP double mutants E73A/Y50A, E73A/Y143A, and Y50A/Y143A and testing the mutants for inhibition of each of the four class A β-lactamases. The coupling free energy ($\Delta G_C$) was used to evaluate the independence of the mutations (40–42). In an additive effect, $\Delta G_C$ will be near zero, which means that the two residues act independently of one another. Considering experimental errors, an
additive effect was defined when $\Delta G_r$ ranged from $-1.00$ to $1.00$ kcal/mol.

As discussed above, SHV-1 is 68% identical in amino acid sequence to TEM-1 $\beta$-lactamase and yet binds BLIP $\sim$2000-fold weaker than TEM-1. The alanine mutagenesis data indicates that residues in the second domain of BLIP that contribute to the binding of TEM-1 $\beta$-lactamase, such as Trp-112 and Trp-150, do not contribute as substantially to binding SHV-1 $\beta$-lactamase. It was also found that some substitutions, such as Y143A, bind SHV-1 much tighter than wild-type BLIP. A possible explanation for these observations is negative cooperativity between residues in the BLIP binding interface. By this view, the presence of a certain residue at one site prevents an optimal interaction with the target protein by a residue at another site at the interface (43). If there exists negative cooperativity between Tyr-143 and Trp-112 or Trp-150, the double mutants W112A/Y143A and W150A/Y143A should exhibit a less than additive effect on binding than the sum of the single mutants. As seen in Table III, the effect of the W112A/Y143A double mutant is similar to the sum of the effects of the single mutants, suggesting these positions are independent, and therefore, the increase in binding affinity of the Y143A substitution is not mediated through Trp-112. In contrast, the W150A/Y143A binds with $-1.0$ kcal less energy than predicted from the sum of the single mutants. This suggests that Trp-150 makes stronger interactions with SHV-1 in the background of the Y143A mutation, which suggests that Tyr-143 is responsible in part for the weak interaction between the second domain of BLIP and SHV-1. In addition, double mutants containing Glu-73 display clearly non-additive effects with respect to binding to SHV-1. For example, based on the single mutant results, the E73A/Y50A double mutant is expected to bind much tighter than what is observed. Similarly, the E73A/Y143A double mutant binds SHV-1 with significantly less affinity than expected based on the sum of the single mutants. These results suggest the presence of Glu-73 prevents optimal interactions between Tyr-50 and Tyr-143 with SHV-1 $\beta$-lactamase. The Glu-73 double mutants also bind Bla1 $\beta$-lactamase with significantly lower affinity than expected based on the sum of the single mutants, suggesting that Glu-73 also restricts interactions between Tyr-50 and Tyr-143 with Bla1 (Table III). In contrast, the Glu-73 double mutants do not exhibit strong non-additivity effects with respect to binding the TEM-1 and SME-1 $\beta$-lactamases (Table III). Examination of the $\beta$-lactamase sequence alignment (Table I) for the region contacted by Glu-73 suggests that the presence of Asp-104 in SHV-1 and Bla1 versus Glu and Tyr at position 104 in the TEM-1 and SME-1 enzymes may contribute to the observed differences in affinity relationships for Glu-73 interactions with the target $\beta$-lactamases.

Functional Epitopes for $\beta$-Lactamase Binding.—The common functional epitope of BLIP for binding the four class A $\beta$-lactamases TEM-1, SME-1, SHV-1, and Bla1 consists of only 5 residues, including Phe-36, His-41, Asp-49, Tyr-50, and Tyr-53. Four additional residues in domain 2 of BLIP, Trp-112, His-148, Arg-160, and Trp-162, make energetically significant contributions to binding of all of the $\beta$-lactamases except SHV-1. The lack of contribution of these four residues for binding SHV-1 may explain the low affinity between BLIP and this enzyme. Therefore, the nine residues discussed above are considered as common affinity determinants for tight binding to class A $\beta$-lactamases. These residues, with the exception of Asp-49, make mainly hydrophobic interactions with target enzymes. Important residues from domain 1 (Phe-36, His-41, and Tyr-53) form hydrophobic interactions with Pro-107–Val-108 of TEM-1 $\beta$-lactamase. Pro-107 is conserved through all class A $\beta$-lactamases that are inhibited by BLIP, and most of these enzymes have a valine or isoleucine at position 108. Both residues 107 and 108 in TEM-1 $\beta$-lactamase were previously shown to be important for BLIP binding (28). The fact that these hydrophobic interactions are important for binding between class A $\beta$-lactamases and BLIP is consistent with previous systematic analyses of protein-protein interfaces (14).

Glu-31, Gly-48, Gln-141, Arg-144 and four serine residues of BLIP do not make significant contributions to binding affinity for any of the tested $\beta$-lactamase targets based on the alanine substitution results (Table II). These residues are mainly polar and located at the periphery of the interface. Clackson and Wells have proposed that peripheral residues might contribute to binding specificity by repulsion of non-target molecules (2). Because these peripheral residues in BLIP do not effect binding affinity to any of the targets tested, they do not appear to play a role in discrimination among class A enzymes; however, they could play a role in the discrimination among natural in vivo targets of BLIP.

The ability to engineer protein-protein interactions or design binding sites could facilitate the development of new therapeutics. A rational design strategy requires understanding of the determinants of affinity in protein-protein interfaces. The presence of hotspots may be a general feature of protein-protein interactions, as suggested by systematic analysis of alanine-scanning mutagenesis data (11). However, the nature of a hot spot is still unclear because of the variation of many physicochemical and geometric parameters of residues in hotspots (4). It is not obvious which residues are critical for binding by examination of structural data. Compounding the challenge is the cooperativity of interactions between residues in protein-protein interfaces (43). For example, the double mutant cycle data presented here indicate that Glu-73 exhibits negative cooperativity with Tyr-50 and Tyr-143 of BLIP with respect to binding SHV-1 and Bla1 but acts independently with regard to binding to the TEM-1 and SME-1 $\beta$-lactamases. The low affinity of the BLIP-SHV-1 interaction appears to result from weak interactions between residues in domain 2 of BLIP, which may be due to negative cooperativity with residues in the specificity-determining region. It is also interesting to note that three targets: TEM-1, SME-1, and Bla1 $\beta$-lactamase, are inhibited by BLIP at a similar high affinity even though residues in the specificity-determining region exhibit widely different effects upon mutation to alanine. The co-structural crystals of BLIP and class A $\beta$-lactamases in addition to TEM-1 are required for a detailed understanding of the molecular basis for interactions in these interfaces.

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