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Fine mapping of the sequence requirements for binding of β -lactamase inhibitory protein (BLIP) to TEM-1 β -lactamase using a genetic screen for BLIP function

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Abstract

β -lactamase inhibitory protein (BLIP) binds and inhibits a diverse collection of class A β -lactamases with a wide range of affinities. Alanine-scanning mutagenesis was previously performed to identify the amino acid sequence requirements of BLIP for binding the TEM-1, SME-1, SHV-1 and Bla1 β -lactamases. Twenty-three BLIP residues that contact TEM-1 β -lactamase in the structure of the complex were mutated to alanine and assayed for inhibition (K_i) of β -lactamase to identify two hotspots of binding energy. These studies have been extended by the development of a genetic screen for BLIP function in *E. coli*. The *bla*_{TEM-1} gene encoding TEM-1 β -lactamase was inserted into the *E. coli pyrF* chromosomal locus. Expression of wild-type BLIP from a plasmid in this strain resulted in a large decrease in ampicillin resistance while introduction of the same plasmid lacking BLIP had no effect on ampicillin resistance. In addition, it was found that when the BLIP alanine-scanning mutants were tested in the strain, the level of ampicillin resistance was proportional to the K_i of the BLIP mutant. These results indicate that BLIP function can be monitored by the level of ampicillin resistance of the genetic test strain. Each of the 23 BLIP positions examined by alanine-scanning was randomized to create libraries containing all possible substitutions at each position. The genetic screen for BLIP function was used to sort the libraries for active mutants and DNA sequence analysis of functional BLIP mutants identified the sequences required for binding TEM-1 β -lactamase. The results indicated the BLIP surface is tolerant of substitutions in that many contact positions can be substituted with other amino acid types and retain wild type levels of function.

Keywords

enzyme inhibition; molecular recognition; antibiotic resistance; random mutagenesis

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Introduction

Protein-protein interactions are critical for most biological processes and a detailed understanding of the molecular basis of these interactions may lead to accurate methods to predict existing interactions and engineer new interactions. Computational prediction and design of protein-protein interaction sites is difficult because they are large ($>900\text{\AA}^2$) and relatively flat¹. In addition, the binding energy within the interaction site between proteins is not evenly distributed among contact residues. Several protein interfaces have been shown to be made up of “hotspot” residues that make a large contribution to binding energy along with other residues that contribute little to the binding energy of an interface^{2; 3; 4}. It is not currently possible, however, to accurately predict the energetic contribution of interface residues or to design new protein-protein interfaces⁵. The accumulation of detailed information on structure-function relationships in protein-protein interfaces should contribute to the understanding necessary for high resolution prediction and design of protein interfaces.

β -lactamases catalyze the hydrolysis of β -lactam antibiotics to provide bacteria with resistance to these drugs. They are grouped into 4 classes (A, B, C and D) according to amino acid sequence homology^{6; 7}. Enzymes from classes A, C and D are serine hydrolases while class B enzymes are zinc metallo-enzymes unrelated in structure to the other classes⁸. Class A β -lactamases exhibit broad substrate hydrolysis profiles which include penicillins, cephalosporins and, for a few enzymes, carbapenems^{8; 9}. TEM-1 β -lactamase is a class A enzyme and is the most common plasmid-encoded β -lactamase in Gram-negative bacteria and as a consequence is a widespread source of antibiotic resistance^{10; 11}. TEM-1 β -lactamase efficiently hydrolyzes penicillins and many cephalosporins but it is not an effective catalyst for extended-spectrum cephalosporins. In addition, mechanism-based inhibitors of TEM-1 β -lactamase such as clavulanic acid are used in conjunction with penicillins to avoid this resistance mechanism. Nevertheless, TEM-1 β -lactamase-mediated resistance has evolved over the past several years due to mutations in the *bla*_{TEM-1} gene which result in amino acid substitutions that allow the TEM enzyme to hydrolyze extended-spectrum cephalosporins or to avoid the action of mechanism-based inhibitors¹².

BLIP is an acronym for β -lactamase inhibitory protein¹³. BLIP is a 165 amino acid protein which binds and inhibits several class A β -lactamases including TEM-1 β -lactamase^{4; 13; 14}. BLIP is produced by the soil bacterium *Streptomyces clavuligerus*¹³. This organism also produces the small molecule, mechanism-based inhibitor clavulanic acid¹⁵. Gene knockout studies indicate BLIP is not essential for growth and the exact role of BLIP in the biology of *S. clavuligerus* remains unknown¹⁶.

BLIP is known to bind to a diverse set of class A β -lactamases with a wide range of affinities^{4; 14}. For example, it binds to TEM-1 β -lactamase with a K_i of 0.5 nM but binds to the SHV-1 β -lactamase, which is 68% identical in amino acid sequence to TEM-1, with a K_i of 1.1 μ M¹⁷. In addition, high-resolution co-crystal structures of BLIP in complex with TEM-1 and SHV-1 β -lactamase are available^{18; 19}. The BLIP- β -lactamase interface has been extensively studied using structural, computational and biochemical approaches^{18; 19; 20; 21; 22; 23; 24; 25}. Extensive site-directed mutagenesis experiments by Schreiber and colleagues suggest the interface consists of modules of TEM-1 and BLIP residues where cooperative interactions occur between residues within modules and additive interactions occur among residues in different modules^{26; 27}.

In addition to the above studies, we previously performed alanine-scanning mutagenesis to identify the amino acid sequence requirements of BLIP for binding the TEM-1, SME-1, SHV-1 and Bla1 β -lactamases^{4; 17; 28}. Twenty-three BLIP residues that contact TEM-1 β -lactamase based on the X-ray structure were mutated to alanine and assayed for inhibition (K_i) of β -

lactamase. This work identified three regions, including two loops that insert into the active site of TEM-1 β -lactamase and a Glu73-Lys74 buried charge motif as being important determinants of the specificity of binding to various β -lactamases^{4; 28}. In addition, two sets of hotspot residues were found to be common sources of binding energy for BLIP interactions with several class A β -lactamases⁴.

Alanine-scanning mutagenesis provides information on the contribution of residue positions to protein-protein interactions but does not provide a detailed view of the sequence requirements with respect to the twenty naturally occurring amino acids found in proteins²⁹. However, constructing, purifying and testing all 19 substitutions at each of the 23 BLIP residues that contact β -lactamase would be a daunting task. An alternative to testing individual substitutions is to randomize the codon of a residue position to form a library of substitutions that can then be tested in batch for those substitutions that retain function. The codon randomization approach requires a rapid method to sort the libraries for functional clones such as a genetic selection or a powerful screen such as phage display^{23; 30; 31}. In this study, a genetic screen for BLIP function in *E. coli* was developed and used to identify BLIP mutants that retain the ability to bind TEM-1 β -lactamase. A total of 23 libraries were generated by randomizing the codon for each of the BLIP residues that contact TEM-1 β -lactamase and the libraries were screened for binders. Two data sets were established. One set included mutants that had partial function while the other set were more stringently screened for high levels of function. The results indicate that a correlation between previous alanine scanning results and the randomization results exists for the stringently selected set and is apparent but weak for the partial function mutants. The randomization approach coupled with the genetic screen is complementary to alanine scanning and taken together the results provide a detailed description of the sequence requirements for BLIP function.

Results

Development of a genetic screen for BLIP function

The goal of this study was to use saturation mutagenesis to obtain detailed information on the sequence requirements at BLIP residues that contact TEM-1 β -lactamase. In order to rapidly test large numbers of mutants it is very useful to have a genetic selection or screen with which to evaluate function *in vivo*. In order to develop a genetic screen for BLIP function, the gene and promoter for TEM-1 β -lactamase was placed on the chromosome of *E. coli* within the *pyrF* gene using recombineering methods to create strain *E. coli* TP112³² (Materials and Methods) (Figure 1). The advantage of placing the *bla*_{TEM-1} gene on the chromosome with its constitutive, weak promoter is that it limits the copy number of the gene to one. It is important for the performance of the screen that the total amount of BLIP be greater than the amount of β -lactamase in the cell. If the number of β -lactamase molecules is higher than the number of BLIP molecules, even very tight binding BLIP variants will not result in a large reduction in β -lactam resistance levels because excess β -lactamase will be available to hydrolyze antibiotic. The insertion within the *pyrF* gene, which encodes orotidine 5'-monophosphate decarboxylase and is involved in pyrimidine biosynthesis, results in the *E. coli* strain being unable to grow on minimal medium. This phenotype can be used as a secondary screen to test for insertion of the *bla*_{TEM-1} gene into the chromosome.

In order to establish the screen, the pGR32 plasmid¹⁷ encoding BLIP fused to the β -lactamase signal sequence and containing a His-tag sequence and under the transcriptional control of the *trc* promoter was introduced into *E. coli* TP112 by transformation. This plasmid was previously used for alanine-scanning mutagenesis of BLIP and the protein is known to be expressed in a functional form^{4; 17; 28}. As a negative control, the parent plasmid, pTP123, which lacks the BLIP gene, was also introduced into *E. coli* TP112¹⁷. Since both BLIP and TEM-1 β -lactamase contain signal sequences and have previously been shown to be exported to the periplasm in

E. coli 17, it was expected that they would be secreted to the periplasm in this construct. In this system, if BLIP is functional, it will bind β -lactamase and impair the ability of the enzyme to hydrolyze ampicillin, thus reducing the ampicillin resistance levels of *E. coli*. The minimal inhibitory concentration (MIC) for ampicillin was determined for *E. coli* TP112 containing pGR32 and, independently, pTP123. It was found that the ampicillin MIC was greatly reduced for the strain containing pGR32 encoding BLIP (1.5 μ g/ml) versus that containing pTP123 with no BLIP (256 μ g/ml) (Table 1). These results suggest that the BLIP is functional *in vivo* and that a genetic screen for that function has been established.

In previous studies, several alanine substituted BLIP mutants were constructed in the pGR32 plasmid and the expressed proteins were purified and tested by measuring the inhibition constant, K_i , for TEM-1 β -lactamase mediated antibiotic hydrolysis^{4; 17; 28}. These mutants were introduced into *E. coli* TP112 to test the relationship between the change in binding free energy of each alanine mutant ($\Delta\Delta G$, derived from the K_i values) and the ampicillin resistance levels provided by the mutant (expressed as log MIC in Fig. 2). As seen in Table 1 and Figure 2A, there is a correlation ($r^2 = 0.49$) between ampicillin resistance levels and the $\Delta\Delta G$ of the BLIP mutants, with tight binding mutants exhibiting low ampicillin MICs and weaker binding mutants exhibiting progressively higher ampicillin MICs. These results suggest that the ampicillin resistance level is inversely proportional to BLIP affinity for binding β -lactamase and therefore that the genetic screen can be used to sort mutant libraries for BLIP variants that retain tight binding to β -lactamase ($\Delta\Delta G \sim 0$).

Construction and screening of BLIP random libraries

As stated above, alanine scanning mutagenesis of 23 BLIP residues was previously used to identify those residues that contribute the most to binding energy in the complex^{4; 28}. In order to gain more detailed information about the chemical side chain requirements for binding, the codons for each of these residues were randomized individually by overlap extension PCR to create 23 libraries, each of which contains all possible substitutions for the position randomized³³ (Materials and Methods). Each library was sorted using the genetic screen for those mutants that retain BLIP function. Because the goal is to find clones with high levels of BLIP function, it was necessary to screen a large number of mutants to identify those exhibiting low ampicillin resistances, which is reflective of strong inhibition of β -lactamase. This was accomplished by transforming each library into *E. coli* TP112 containing TEM-1 β -lactamase encoded on the chromosome. Approximately ten colonies were picked from each library without screening for ampicillin resistance to ensure the libraries were not obviously biased (Fig. 3A). In order to screen for ampicillin resistance, individual transformants were picked and inoculated into growth medium containing various concentration of ampicillin in 96-well microtiter plates. Mutant clones that exhibited low levels of ampicillin resistance were chosen and tested for their ampicillin minimal inhibitory concentration (MIC) (Materials and Methods). Those mutants that exhibited resistance levels similar to *E. coli* TP112 containing the wild type His-tagged BLIP (<4 μ g/ml MIC) were analyzed by DNA sequencing to determine the identity of the amino acid substitution at that position. This process was repeated for each of the 23 libraries and the results are shown in Figure 3B.

It is apparent from DNA sequencing results that the majority of the 23 contact positions are quite tolerant of amino acid substitutions. For example, Asp49 is a key BLIP residue whose side chain is positioned in the TEM-1 β -lactamase active site where it makes several contacts with active site residues¹⁹. When functional clones from the Asp49 library were identified and sequenced it was found that several types of hydrophilic substitutions were consistent with function (Fig. 3B). In addition, an alanine substitution was identified at this position among the functional clones. It is known from previous studies, however, that that Asp49Ala substitution has a K_i of 20 nM, which is 40 times higher than the wild type BLIP K_i of 0.5 nM

(Table 1)28. This result suggested that the genetic screen identifies mutants with wild type function as well as mutants with partial function. Examination of the correlation between K_i and ampicillin MIC of the alanine mutants in Table 1 suggests that mutants with ampicillin MIC values in the same range as wild type BLIP (1–4 $\mu\text{g/ml}$) show a range of K_i values from 0.01 to 46 nM. These mutants could all be considered tight binding in that the binding constants are in the low nanomolar range, however, they do not all function as efficiently as wild type. This finding was further confirmed by purifying and determining the K_i value for inhibition of TEM-1 β -lactamase by several of the BLIP mutants listed in Figure 3 including the Glu31Gln, Asp49Ser, Glu73Tyr, Trp112Arg, and Phe142Gly proteins (Table 2). The K_i values ranged from 1.4 to 16 nM, consistent with the hypothesis that these mutants bind tightly to TEM-1 β -lactamase (<20 nM) but have partial function relative to wild type His-tagged BLIP ($K_i = 0.5$ nM).

It is expected that the ampicillin MIC of an *E. coli* strain containing BLIP and TEM-1 β -lactamase would be sensitive to the relative amounts of these proteins which, in turn, is determined by the expression levels of these proteins. The pGR32 plasmid used for BLIP expression in this and previous studies is under the transcriptional control of the *trc* promoter¹⁷. This promoter is a fusion of the *trp* and *lac* promoters and is IPTG-inducible³⁴. The pGR32 expression plasmid also encodes the *lacI* repressor which down-regulates the promoter in the absence of IPTG³⁴. The genetic screen experiments described above were performed in the absence of IPTG and therefore the BLIP expressed is due to basal or “leaky” expression. The *E. coli* TP112 strain used for the screen contains the *lacX74* deletion which removes the entire *lac* operon, including *lacI*. Therefore, the levels of basal expression of BLIP from the *trc* promoter could be relatively high in the *E. coli* TP112 strain. In an effort to gain tighter control of BLIP expression in the absence of IPTG, the *bla*_{TEM-1} gene was moved to the chromosome of *E. coli* MG1655 by P1 transduction and the new strain was named *E. coli* TP132. This strain contains no mutations in the *lac* operon or control region and it was reasoned that basal BLIP expression levels relative to β -lactamase may be reduced in this strain, which would result in a more stringent requirement for BLIP function.

The relationship between ampicillin MIC and K_i for inhibition of TEM-1 in *E. coli* strain TP132 was examined using the set of BLIP alanine-scanning mutants as described above. It is apparent from the results in Figure 2B and Table 1 that there is a stronger correlation ($r^2 = 0.76$) between ampicillin MIC levels and the K_i inhibition constants and the corresponding $\Delta\Delta\text{G}$ values. The stronger correlation is also indicated by more stringent sequence requirements found for BLIP mutants in *E. coli* TP132. For example, among the Asp49 mutants identified in the screen using *E. coli* TP112, only the Asp49Glu mutant exhibited ampicillin MICs similar to wild type in the *E. coli* TP132 strain (Figure 3B). In addition, the ampicillin MIC of 12 $\mu\text{g/ml}$ of the W112R mutant with a K_i of 3.9 nM is higher than the 4 $\mu\text{g/ml}$ ampicillin MIC for wild type BLIP that has a K_i of 0.5 nM (Table 2). Furthermore, the ampicillin MIC results for 13 BLIP alanine mutants in *E. coli* TP112 and TP132 were compared to $\Delta\Delta\text{G}$ binding energy determinations for these mutants published by Reichmann et al.²⁶ (Materials and Methods). This comparison is of interest because the mutants are a subset of those listed in Table 1 and because the binding energies were evaluated by surface plasmon resonance (SPR) rather than inhibition assays²⁶. As seen in Figure 2C and D, the correlations between the change in binding energy of the mutants determined by SPR versus the ampicillin MIC values are very similar to those obtained using $\Delta\Delta\text{G}$ values derived from K_i values for both *E. coli* TP112 ($r^2 = 0.46$) and TP132 ($r^2 = 0.79$). Taken together, the results indicate that determining the ampicillin MIC of mutants in the *E. coli* TP132 strain is a useful method to identify mutants with binding constants similar to wild type BLIP.

In order to gain more detailed information on the sequence requirements for BLIP function, each of the mutants identified in the initial genetic screen in *E. coli* TP112 (bottom panel of

Fig. 3B) were introduced into the *E. coli* TP132 strain and the ampicillin MIC of the resultant strains was determined to more accurately identify those mutants with wild type levels of function (Fig. 3, 4). Those mutants that exhibited wild type levels of ampicillin resistance in *E. coli* TP112 but increased levels of resistance in *E. coli* TP132 are assumed to have sub-wild type BLIP inhibition function and are not listed in the top panel of Figure 3B. As seen in the top panel of Figure 3B, the sequence requirements for high level function are, in general, more stringent than those required for partial function. The tolerance of the BLIP residue positions to amino acid substitutions follow the same general trends in both experiments but, for several positions, fewer substitutions are consistent with the very tight binding of the wild type BLIP (0.5 nM). It is also of interest that some positions did not exhibit a large difference in substitution patterns in the screen for partial or wild type levels of function. For example, residues Tyr53 and Trp150 were found to be relatively intolerant of substitutions in both screens suggesting that nearly all substitutions at these positions result in a large decrease in function and that there is a precise requirement for the physical-chemical properties of the side chains at these positions. The sequence requirements at residues Glu31, Ser35, Tyr50, Tyr51, Ser71, Trp112, Ser113, Tyr143, Arg160 and Trp162 also did not change significantly between screens in that several different amino acids can substitute at these positions without impacting function. This finding suggests that a wide range of chemical properties of side chains are consistent with function at these positions. Finally, several positions did exhibit a significant difference in sequence requirements for partial versus wild type levels of function. These positions include Asp49, where only Asp and Glu exhibit wild type levels of function in the stringent screen while several different substitutions are consistent with partial function (Fig. 3B). Similarly, positions Phe36, His41, Ser39, Gly48 and Phe142 exhibit stringent sequence requirements for wild type (*E. coli* TP132) versus partial levels (*E. coli* TP112) of function. The pattern of substitutions between the screens for these residues suggests that there are stringent requirements on the chemical nature of the side chains for the tight binding characteristic of wild type BLIP but that more diverse chemical properties are consistent with partial function (< 20 nM K_i).

Discussion

In this study, a genetic screen for BLIP function was developed and validated using a set of previously characterized alanine substitution mutants of BLIP⁴. The availability of quantitative enzyme inhibition data for the alanine mutants allowed optimization of the system to modulate the levels of BLIP function required for mutants to pass the screen. The 23 BLIP residue positions that are in contact with TEM-1 β -lactamase based on the crystal structure and for which alanine mutants had previously been examined were randomized individually to create libraries containing all possible amino acid substitutions at each position. Each library of mutants was examined using the genetic screen to identify clones with ampicillin MIC values similar to a control strain encoding wild type BLIP. The clones with wild type levels indicate sequences that are consistent with tight binding to β -lactamase. The results revealed a wide range in sequence requirements among the 23 contact positions.

Eight residue positions in BLIP including Phe36, Ser39, His41, Gly48 Asp49, Tyr53, Phe142 and Trp150, exhibited very stringent sequence requirements with only one other amino acid besides wild type being consistent with high level function. For all of these positions, the one substitution was chemically conservative compared to the wild type residue (Figure 3B). Therefore, these residue positions have precise sequence requirements in order to provide tight binding BLIP function. It should be noted, however, that the sequence requirements could be due to protein stability requirements as well as binding requirements. The positions mutated are on the surface of the protein and so destabilizing substitutions are less likely but the *in vivo* screen does not distinguish binding versus stability defects. The randomization and selection results for positions Phe36, His 41, Asp49, Tyr53, Phe142 and Trp150 are consistent

with the previous alanine scanning mutagenesis results in that alanine substitutions at each of these residues resulted in a greater than 20-fold loss in binding affinity as indicated by the inhibition constant for TEM-1 β -lactamase^{4; 28}. Among these residues, Asp49 and Phe142 are present on loop structures in BLIP and both insert into the active site of TEM-1 β -lactamase. Asp49 makes numerous interactions with TEM-1 active site residues and appears to mimic the interactions of the carboxylate group found in β -lactam antibiotics¹⁹. Phe142 fills a section of the active site in a region similar to that occupied by the benzyl group of benzylpenicillin¹⁹. Therefore, there appear to be precise volume and chemical composition restrictions on these positions which greatly restrict the amino acids that can substitute for the wild type residues. Residues Phe36 and Trp150 are large aromatic residues that are near the periphery of the binding site and flank each side of a TEM-1 β -lactamase loop region containing residues 99-112 and appear to act as important “clamps” on the loop. Tyr53 is buried in the BLIP-TEM-1 β -lactamase interface approximately 6 Å from Phe36 where it interacts with Pro107 from TEM-1 presumably to also stabilize interactions with the 99-112 loop of TEM-1. Previous results indicated that an alanine substitution at His41 resulted in a greater than 50-fold loss in binding affinity as measured by the inhibition constant^{4; 28} and the stringent sequence requirements are consistent with this observation. His41 is situated between the critical BLIP residues Phe36 and Tyr53 where it contributes to the surface of BLIP that interacts with the 99-112 loop of TEM-1 in the vicinity of TEM-1 Pro107.

BLIP positions Ser39, Gly48 and Gly141 also exhibited stringent sequence requirements. Interestingly, alanine substitutions at Ser39, Gly48 and Gly141 had little effect on binding affinity and yet these positions are not tolerant of substitutions^{4; 28}. This is due the ability of small volume amino acids to substitute these positions (Fig. 3B). Ser39 forms a cluster with Phe36, Tyr53 and His41 and interacts with side chain of Lys111 in the 99-112 loop of TEM-1. Gly48 and Gly141 appear to play similar roles in that they are adjacent to Asp49 and Phe142 that are on the BLIP loops that insert into the β -lactamase active site. The sequence constraint to small amino acids may be due to steric factors on the turn structures on which Asp49 and Phe142 reside. It is unclear why alanine mutants were not observed among the functional mutants for Ser39 and Gly141 since alanine did not greatly affect the K_i values. This could be due to a failure to sample the alanine mutant or these could be false negatives due to altered protein expression levels of BLIP mutants.

Several residues in BLIP were previously found to contribute strongly to binding via alanine substitution experiments and yet could be substituted by several different amino acids in the randomization experiments and retain function. These positions include Lys74, Trp112, Arg160 and Trp162. Lys74 is an interesting position in that it appears to play a key role in mediating the binding specificity of BLIP because substitution of this residue by alanine results in a greater than 50-fold loss in binding affinity for TEM-1 but a 10-fold increase in binding affinity for SME-1 β -lactamase^{4; 28}. In the BLIP-TEM-1 β -lactamase structure, Lys74 interacts with Glu104 from TEM-1 to form a key buried salt bridge which is consistent with the finding that substitution by alanine reduces affinity¹⁹. Nevertheless, several different amino acid types are found at position 74 among the random mutants with high levels of BLIP function (Fig. 3B). The arginine and histidine substitutions may be able to approximate the positive charge of the lysine interaction to retain function. However, charge does not appear to be essential since leucine and tyrosine are also found among the functional mutants.

The Trp112 residue in BLIP is buried in the complex near the center of the binding interface between BLIP and the 99-112 loop region of TEM-1 β -lactamase¹⁹. Substitution of Trp112 with alanine results in a loss of binding affinity with TEM-1 as well as with all other class A β -lactamases tested including SME-1, SHV-1 and Bla1⁴. Despite this fact, several different amino acid types were found at position 112 among random mutants with BLIP function (Fig.

3B). Several of the substitutions such as leucine, isoleucine and valine are conservative and may retain similar interactions; however the serine substitution is more difficult to rationalize.

Substitution of BLIP Arg160 with alanine also results in a large loss of binding affinity for TEM-1 as well as SME-1, SHV-1 and Bla1 β -lactamases⁴. However, several amino acid types at position 160 are consistent with BLIP binding to TEM-1 β -lactamase. Similarly, substitution of Trp162 in BLIP with alanine results in a greater than 20-fold loss in binding affinity to TEM-1 β -lactamase but the position is substituted by several residue types among functional mutants (Fig. 3B). Trp162 is near the periphery of the interface between BLIP and the TEM-1 99-112 loop region where it packs against TEM-1 residues Val103 and Pro167¹⁹. Substitution of Trp162 with phenylalanine and histidine could reconstitute these interactions but the arginine and glutamine substitutions may work by a different mechanism (Fig. 3).

As is apparent from the above discussion, there is general agreement between the results obtained by alanine scanning mutagenesis versus the randomization and selection strategy but there are several exceptions. This is indicated in Fig. 5, which shows the $\Delta\Delta G$ of the alanine mutant for each position versus the number of amino acids that can be substituted at each position in the more stringent *E. coli* TP132 strain. Note that the exceptions, including positions Lys74, Trp112, Arg160 and Trp162, lie above the correlation line, i.e., $\Delta\Delta G$ for the alanine substitution is large and positive but the position can be substituted by several amino acids. From these results it is apparent that the saturation mutagenesis results provide a more fine-grained view of the sequence requirements for protein function.

Taken together, the randomization results in Fig. 3 indicate that the BLIP binding surface is quite tolerant of mutations. With the exception of one position, the residues analyzed are tolerant of at least one amino acid substitution and for most positions the presence of any of several different amino acids is consistent with function. A recent comprehensive saturation mutagenesis study to examine the sequence requirements for binding of human growth hormone (hGH) to human growth hormone receptor also found that residues in the hGH binding surface were tolerant of substitutions³¹. In addition, the hGH study found that, for several positions, the substitutions observed were not conservative in chemical type³¹. Similar observations were made in this work for a subset of residues including Lys74, Trp112, Arg160 and Trp162 as described above (Fig. 3). Therefore, the hGH-hGHR and BLIP- β -lactamase interfaces exhibit some similarities in terms of overall sequence requirements.

The accurate computational prediction of binding energy in protein-protein interactions is an important goal for protein engineering and design as well as the protein docking studies. Several structure-based computational methods have been developed to estimate free energy changes associated with mutations^{35; 36; 37; 38; 39}. As a first step towards comparing the BLIP experimental results with computational predictions, the results of the genetic screen in Fig. 3 were compared with free energy change predictions made using the FoldX force field^{36; 40; 41}. The free energy changes in the BLIP-TEM-1 β -lactamase complex were calculated using FoldX for all 19 mutants for each of the 23 BLIP positions that were randomized in this study (Materials and Methods). Although, there was variation in the extent of correlation from position to position, it was found that the average calculated $\Delta\Delta G$ for binding for the mutants from all 23 libraries with high levels of BLIP function (low ampicillin MIC) identified in the genetic screen in *E. coli* TP132 (Fig. 3B, top panel) was 2.93 kJ/mol. In contrast, the average calculated $\Delta\Delta G$ for all possible substitutions for all positions was 6.62 kJ/mol. Therefore, as a first approximation, FoldX appears to identify the functional tight binding BLIP mutants (low ampicillin MICs and low calculated $\Delta\Delta G$) from the general pool of mutants. This point is illustrated for each of the 23 randomized positions in Figure 6. With the exception of positions E31, E73 and R160, the averaged FoldX calculated $\Delta\Delta G$ values for the functional mutants identified at each residue position in the genetic screen are lower than the averaged FoldX

calculated $\Delta\Delta G$ values for all 19 mutants at each position (Supplemental Table 1). However, the results in Figure 6 also illustrate those positions where the FoldX calculated $\Delta\Delta G$ values for the functional mutants are relatively high and therefore predict weak binding including residues G48, E73, K74, R144 and R160. It will be of interest to study the binding characteristics of BLIP variants with substitutions at these positions in more detail.

BLIP is known to bind to a number of different class A β -lactamases with a wide range of affinities^{4; 14}. Previous studies of 23 contact residues by alanine substitution followed by quantitation of binding to the TEM-1, SME-1, SHV-1 and Bla1 β -lactamases distinguished three classes of positions, those where alanine substitution did not effect binding to any β -lactamase, which reflect positions whose side chains do not contribute to binding; those where alanine substitution decreased binding to all β -lactamases, which reflect positions that are important for β -lactamase binding in general; and those where alanine substitutions had different effects depending on the β -lactamase being examined, which reflects positions that influence the substrate specificity of BLIP. The results in Figure 5 indicate that the alanine scanning results for BLIP binding TEM-1 do not always correlate with how freely a position can be substituted by saturation mutagenesis. It will be of interest to determine the sequence requirements for BLIP binding to other class A β -lactamases and how these requirements compare to those for binding TEM-1. Such studies should provide additional insights into mechanisms of molecular recognition between related proteins.

Materials and Methods

Bacterial strains and plasmids

The *E. coli* TP112 strain was constructed from the recombinering strain *E. coli* SW102 whose genotype is: F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*dlacZ* M15 *AlacX74 deoR recA1 endA1 araD139* Δ (*ara, leu*) 7649 *galU galK rspL nupG* [*lcI857 (cro-bioA)* $\langle \rangle$ *tet*]³². The *bla*_{TEM-1} gene was introduced on a PCR product containing 35 base pairs (bp) of flanking sequence from the *pyrF* gene on each end. The primers used for amplification were: *pyrF*-*bla*-top 5'-

CACGCGATTGTCGTCTGAAGGTCGGCAAAGAGATGGTTTCTTAGACGTCAGGTG
GCACTTTTCGGGGAAATG-3' *pyrF*-*bla*-bot 5'-

GCGGTGCATCTTTGCCAAACGGAACCAGTGCCTCTTACCAATGCTTAATCAGTG
AGGCACCTATCTCAGCG-3'. The *E. coli* SW102 strain was grown and concentrated for electroporation at 30°C. The *pyrF*-*bla* PCR product was electroporated into *E. coli* SW102 and the cells were spread on agar plates containing 50 μ g/ml ampicillin and 5 μ g/ml tetracycline. The presence of the *bla*_{TEM-1} gene on the chromosome inserted into the *pyrF* gene was validated by PCR amplification of the region followed by DNA sequencing. The genotype of the *E. coli* TP112 strain is: F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*dlacZ* M15 *AlacX74 deoR recA1 endA1 araD139* Δ (*ara, leu*) 7649 *galU galK rspL nupG pyrF::bla*_{TEM-1} [*lcI857 (cro-bioA)* $\langle \rangle$ *tet*]. The *pyrF*-*bla*_{TEM-1} gene region was moved from *E. coli* TP112 to the chromosome of *E. coli* MG1655 (F- *lambda- ilvG- rfb-50 rph-1*) by P1 transduction to create *E. coli* TP132. The genotype of *E. coli* TP132 is: F- *lambda- ilvG- rfb-50 rph-1 pyrF::bla*_{TEM-1}. The BLIP random libraries were introduced into *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'**proAB lacI^qZAM15 Tn10 (Tet^r)*])⁴². *E. coli* RB791⁴³ was used for expression of BLIP mutants for protein purification.

Library construction and screening

The BLIP random libraries were constructed in the pGR32 plasmid which contains BLIP fused to the TEM-1 signal sequence under the transcriptional control of the *trc* promoter¹⁷. The BLIP protein is tagged with a 6x His-tag at the N-terminus of the protein¹⁷. Each of the 23 random libraries was constructed by overlap extension PCR whereby the codon to be

randomized was converted to NNN where N is any of the 4 nucleotides⁴⁴. Briefly, primers complementary to internal and external sequences in the blip gene were used to PCR-amplify two DNA fragments with overlapping ends. Specific mutations in the nucleotide sequence of the internal complementary primers incorporated the NNN sequence at the codon of interest³³. The PCR products were examined on an agarose gel to ensure the reaction occurred to create the expected sized DNA products. The two PCR reactions containing the overlapping fragments were then combined into a single PCR reaction and amplified with the external primers TP123-top and blip-XbaI-4 to create the final PCR fusion product. The PCR product was digested with the *SacI* and *XbaI* restriction endonucleases and ligated into the pTP123 plasmid¹⁷ that had been digested with the *SacI* and *XbaI* enzymes. The ligation products (library DNA) were used to transform *E. coli* XL1-Blue and cells were spread on agar plates containing 12.5 µg/ml chloramphenicol. A minimum of 1000 colonies were pooled for each library. Several clones were picked from each library for DNA sequence analysis to assess the mutagenesis procedure for the libraries. Each random library was used to transform *E. coli* TP112 and cells were spread on agar plates containing 12.5 µg/ml chloramphenicol. Approximately 10 colonies were picked for each library from the chloramphenicol containing agar plates and sequenced to test for any obvious sequence bias in each randomized library in the TP112 strain. Mutant clones with low ampicillin minimum inhibitory concentrations (MICs) were identified by picking individual colonies with toothpicks and inoculating growth media in 96-well microtiter plates. The 96-mini-cultures were diluted into additional 96-well plates containing various concentrations of ampicillin and the plates were grown overnight at 37°C with shaking. Those clones that exhibited low ampicillin resistance in this assay were tested to determine an exact ampicillin MIC using the E-test strip method according to manufacturer's instructions (AB Biodisk). Those clones that exhibited ampicillin MIC values similar to the wild-type His-tagged BLIP control in pGR32 were examined by DNA sequencing to determine the identity of each mutant. The plasmid DNA from each of the sequenced clones with low ampicillin MICs from the *E. coli* TP112 screen was isolated and used to transform *E. coli* TP132. The ampicillin MIC from each of the clones in *E. coli* TP132 was determined by the E-test strip method.

Protein purification and inhibition assays

The wild type, His-tagged BLIP as well as the E31Q, D49S, E73Y, W112R, and F142G mutants were introduced into *E. coli* RB791 and expressed by induction with IPTG. The BLIP proteins were purified by metal-affinity chromatography as previously described¹⁷. TEM-1 β-lactamase was purified using a zinc chelating column as described previously⁴⁵.

The β-lactamase inhibition assays were performed as described previously with minor modifications¹⁷. Briefly, 0.3 nM of TEM-1 β-lactamase was incubated with increasing concentrations purified BLIP and allowed to equilibrate for one hour at 30°C. The colorimetric β-lactam substrate nitrocefin was added to a concentration of 10 µM and the initial velocity of the reaction was monitored at 482 nM. The initial velocity of nitrocefin hydrolysis was plotted versus BLIP concentration and the resulting curve was fit to the equation for a tight binding inhibitor to obtain the inhibition constant K_i as described previously with the modification that the K_i was calculated from the apparent K_i using the equation below as described¹⁸

$$K_i = K_{iapp} / (1 + (S / K_M)).$$

Calculation of ΔΔG and correlation with ampicillin resistance of *E. coli* strains

The K_i values for the 23 BLIP alanine mutants listed in Table 1 were converted to ΔΔG values using the equation $\Delta\Delta G = -RT \ln(K_{iwt}/K_{imut})$. The k_{cal} /mol values thus obtained were

converted to kJoules/mol using 1 calorie equals 4.184 joules. The $\Delta\Delta G$ values for BLIP alanine mutants determined by surface plasmon resonance used for the correlation studies in Fig. 2C and 2D were from Table 1 of Reichmann et al.²⁶. The values used for Fig. 2C and 2D were for BLIP alanine mutants F36A, H41A, D49A, Y50A, Y53A, S71A, K74A, W112A, S113A, F142A, H148A, W150A and R160A²⁶.

Calculation of changes in binding free energy using FoldX

The FoldX 3.0 program package was obtained from the FoldX website (<http://foldx.crg.es>)⁴¹. The 3-D structure of the BLIP-TEM-1 β -lactamase complex (1JTG) was first optimized using the RepairPDB command in FoldX. Each of the 23 BLIP residue positions was mutated to all 19 amino acid substitutions in the A-B complex within 1JTG using the BuildModel command to create a wild type and corresponding mutant PDB file for each substitution at each position. Each PDB file was then evaluated using the AnalyseComplex command to generate a binding interaction energy for each mutant and corresponding wild type structure. The calculated interaction energy of each wild type complex was subtracted from its corresponding mutant to generate a $\Delta\Delta G$ value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Schematic illustration of position of the *bla*_{TEM-1} gene and promoter inserted into the chromosome of *E. coli* TP112 at the *pyrF* locus.

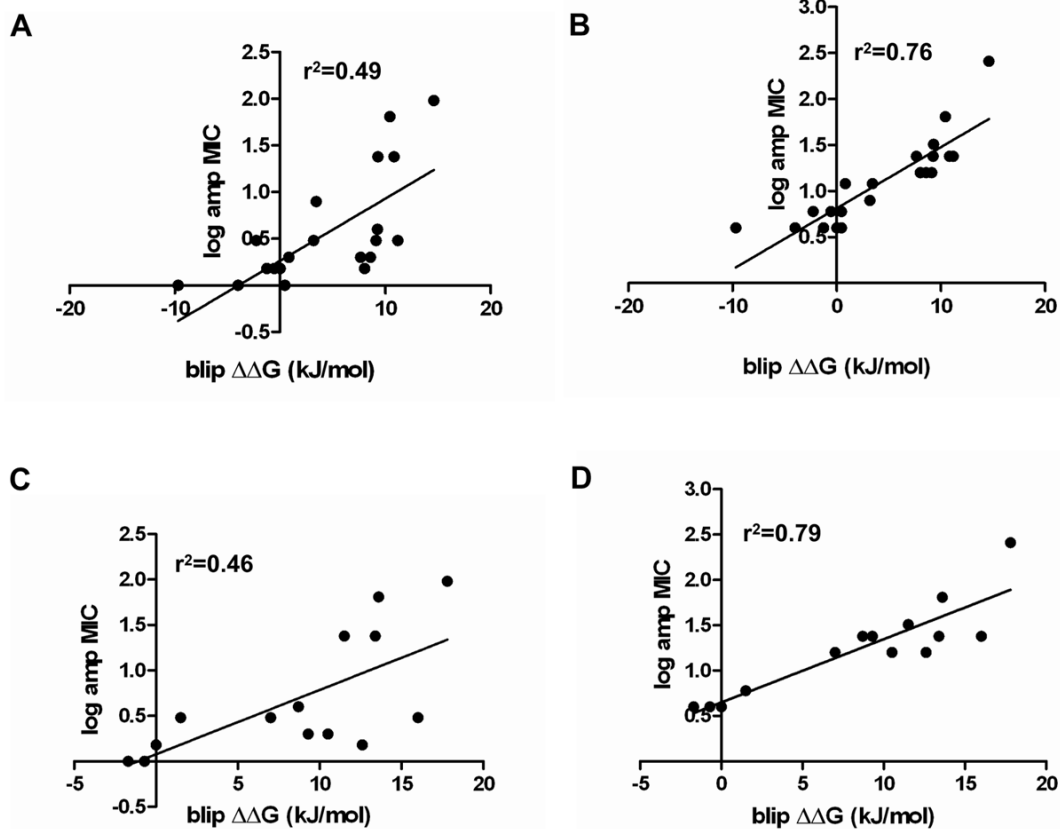


Figure 2.

BLIP alanine mutant ampicillin MIC versus binding energy. Panel A shows a comparison of the log of the minimum inhibitory concentration of ampicillin for growth of *E. coli* TP112 containing plasmids encoding the BLIP alanine mutants versus change in binding energy of the BLIP mutant compared to wild type BLIP ($\Delta\Delta G$ (kJ/mol)) for binding TEM-1 β -lactamase. The $\Delta\Delta G$ values were calculated from the inhibition constants (K_i) and are listed in Table 1. Panel B shows the analogous data using *E. coli* TP132 as the host strain for MIC determinations. Panels C and D use data for 14 BLIP alanine mutants whose $\Delta\Delta G$ values were determined by Reichmann et al. using surface plasmon resonance²⁶. These alanine mutants are a subset of those in Panels A and B and are compared with the corresponding ampicillin MIC data for each mutant in *E. coli* TP112 and TP132. Panel C represents log ampicillin MICs for *E. coli* TP112 and Panel D is log ampicillin MICs for *E. coli* TP132. r^2 is the goodness of the linear regression fit of the indicated line.

A

E31	S35	F36	S39	H41	G48	D49	Y50	Y51	Y53	S71	E73	K74	W112	S113	G141	F142	Y143	R144	H148	W150	R160	W162
CTA-L	CAG-Q	-1fs	AGC-S	GGA-G	TCG-S	GTT-V ²	GGA-G ²	TTC-L ²	AAG-K ²	GGT-G ²	GCG-A	TCA-S	TGG-W ⁴	TCA-S	TAG-*	ATC-M	GAG-E	GTT-V	AAG-K	ACA-T	CGT-R ²	AAG-K
ATT-I	CTC-L	TTG-L	GAC-D	GAG-E	TCA-S	TGA- ¹³	GAT-D	TAC-Y ²	CTT-L	TCA-S	GCC-A	AGG-R ²	CTT-L	CAC-H	TCA-S	ACC-T ²	GTT-V	TTT-F ²	ATA-I	TTA-L ²	CAT-H	GCA-A
AAA-K	TTA-L	TGA-*	TCG-S	GTG-V	GTG-C	AAG-K ²	CCG-P	ATT-I	AGG-R	ATT-I	-1fs	CGT-R	AGA-R	TCT-S	GCA-A	TAA-*	AAT-N	GAA-E	AGT-S	TAC-Y ²	TCA-S	TAC-Y
CCC-P	GAT-D	AGG-R	GGA-G	CCA-P	GGT-G	TAA-*	AAT-N	AGC-S	TTT-F	ACG-T	GTT-V	TGA-*	TTG-L	TCG-S	CGT-R ²	CGC-R	TGA-*	AAC-N	CCT-P	AGG-R	GAC-D	TCC-S
GAA-E	CCC-P	AGT-S	TGG-W	ACT-T	GTG-V	TAG-*	CTC-L	AGT-S	GTC-V	TTC-F	TGA-*	TAA-*	TGA- ²	TAG-*	AAT-N ²	AAG-K ²	-5fs	AGA-R	TCC-S	TGG-W	TTC-L	AGT-S ²
GCT-A	CCG-R	AAG-K	GTT-V	AAG-K	GGG-G	CCT-P	CTA-L	TGA- ²	ACC-T	TGG-W	AGC-S ²	ATT-I	CAC-H	GAG-E ²	CCC-P ²	CTG-L	ATA-I	AAT-N	GAA-E	TAG-*	GGA-G	
AGT-S	ACT-T ²	GGG-G	AAG-K	GGT-G	CCG-P	CGC-R	GAA-E	CAT-H	CTA-L	ATA-I	CCC-P	GGG-G	AGT-S	CAG-Q	GGC-G	ACT-T	GTC-V	CAA-Q	GTA-V	TAT-Y	CAC-H	CTG-L
AAG-K	TCC-S	AAC-N	GCT-A ²	CAC-H	TTG-L ²	CGA-R	GGG-G	GGA-G	GTG-V	ATG-M	TGT-C	TCT-S	GAC-D	ACT-T	ACT-T	GGT-G	-1fs	AAG-K	ACA-T	GAG-E	TTT-F	GAA-E
GTC-V	GAA-E	TAC-Y	TTT-F	ACA-D	GAC-D	ATC-L	GGT-G	CCA-P	TAC-Y	ACA-T	AAA-K	GTT-V	ATC-I	GTT-V	ACT-T	GGT-G	-1fs	CGC-R	GAT-D	TGA-*	AGC-S	GGG-G
AGC-S	GTT-V	-1fs	TTC-F	GAC-D	CAC-H	CTG-L	TTA-L	ACG-T	TAG-*	AGA-R	ACC-T	CTA-L						CGA-R	CCA-P	GTA-V	GGC-G	CTT-L
AGG-R	GCG-A	TCT-S	AAG-K	AAA-fs	TAG-*					TAA-*	GCT-A	TTA-L										
GCA-A		GTG-V																				

B

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E31	S35	F36	S39	H41	G48	D49	Y50	Y51	Y53	S71	E73	K74	W112	S113	G141	F142	Y143	R144	H148	W150	R160	W162																																																																															
Q1.5	S1	F ³ 1.5	S ⁴ 1.5	H1.5	G ⁴ 1.5	D ² 1.5	Y1.5	Y1	Y ³ 1.5	I ³ 1.5	S ² 2	K ² 1.5	W1.5	S ³ 1.5	G ² 1.5	T ³ 1.5	L ³ 2	K ³ 1.5	H1	W ⁶ 1.5	R ² 1	W1																																																																															
M1.5	H1.5	L ⁴ 2	R ³ 2	Y ⁴ 2	A1.5	S ² 2	G ⁵ 1.5	L ³ 1.5	F ⁷ 1.5	F ² 1.5	L2	R ² 1.5	K1.5	R ² 1.5	S ³ 1.5	G ² 1.5	C1.5	N ³ 1.5	L ⁴ 2	Y ² 1.5	S ³ 2	H ⁴ 1.5																																																																															
H ² 1.5	P1	W ² 2	N2	I2	H1.5	Q1.5	A ² 1.5	K1.5	M1.5	W1.5	H2	V2	T1.5	N ² 1.5	I ³ 1.5	S1	D1.5	T ² 1.5	T1.5	R ² 1.5	A1.5	Q1.5																																																																															
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L1.5	L1.5	Y2			E1.5	N ² 2	P1.5		V ³ 1.5	Y1	Y1	A ² 2	Q1.5	K1.5	R1.5	S1.5						F1																																																																															
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Figure 3. Results of DNA sequencing of clones from BLIP libraries before and after screening for ampicillin resistance. A. Nucleotide sequences and corresponding amino acid sequences of clones from each library picked after transformation of libraries into *E. coli* TP112. These clones were picked without screening for ampicillin resistance and are representative of diversity in the libraries. The fs label stands for frameshift mutation. The number before fs indicates the number of nucleotides deleted in the clone. B. Amino acid sequence replacements at BLIP contact positions that retain BLIP binding to TEM-1 β-lactamase as indicated by the genetic screen. Each of the 23 residues shown was individually randomized to NNN where N is any of the 4 nucleotides by site directed mutagenesis to create libraries containing all possible substitutions at each position. Each library was transformed into *E. coli* TP112 (lower panel) and screening was performed using 96-well plates as described in Materials and Methods to identify clones with low ampicillin resistance. Clones with resistance similar to the strain containing wild type BLIP were sequenced and the substitutions are shown below the listed amino acid residue positions. The superscript indicates the number of times a mutant with the listed amino acid was found. The number following the amino acids is the ampicillin MIC (μg/ml) of the clone. The columns above the listed residue positions indicate the substitutions that retain high level function when the ampicillin MIC determinations of the clones from the lower panel were performed in *E. coli* TP132. The number following the amino acids is the ampicillin MIC (μg/ml) of the clone.

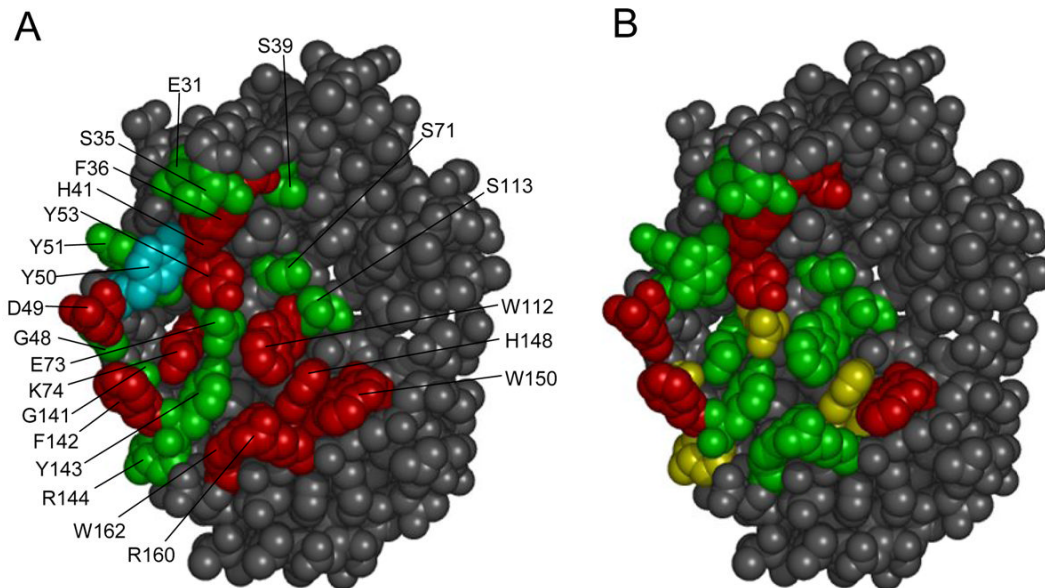


Figure 4.

Structure of BLIP with randomized residues highlighted. Panel A summarizes the results from alanine scanning of BLIP and measurement of binding to TEM-1 β -lactamase²⁸. Residues colored red represent positions where the alanine substitution resulted in a greater than 10-fold loss in binding affinity. Residues colored green represent positions where an alanine substitution had a less than 10-fold effect on binding and the residue colored cyan represents a position where the alanine substitution resulted in a greater than 10-fold increase in binding affinity²⁸. In Panel B the results of the saturation mutagenesis studies are summarized. Positions where 2 or less amino acids substitutions (including wild type) are consistent with function are colored red. Positions where 4 or less amino acids are consistent with function are colored yellow. Positions where greater than 4 amino acid substitutions are consistent with function are colored green.

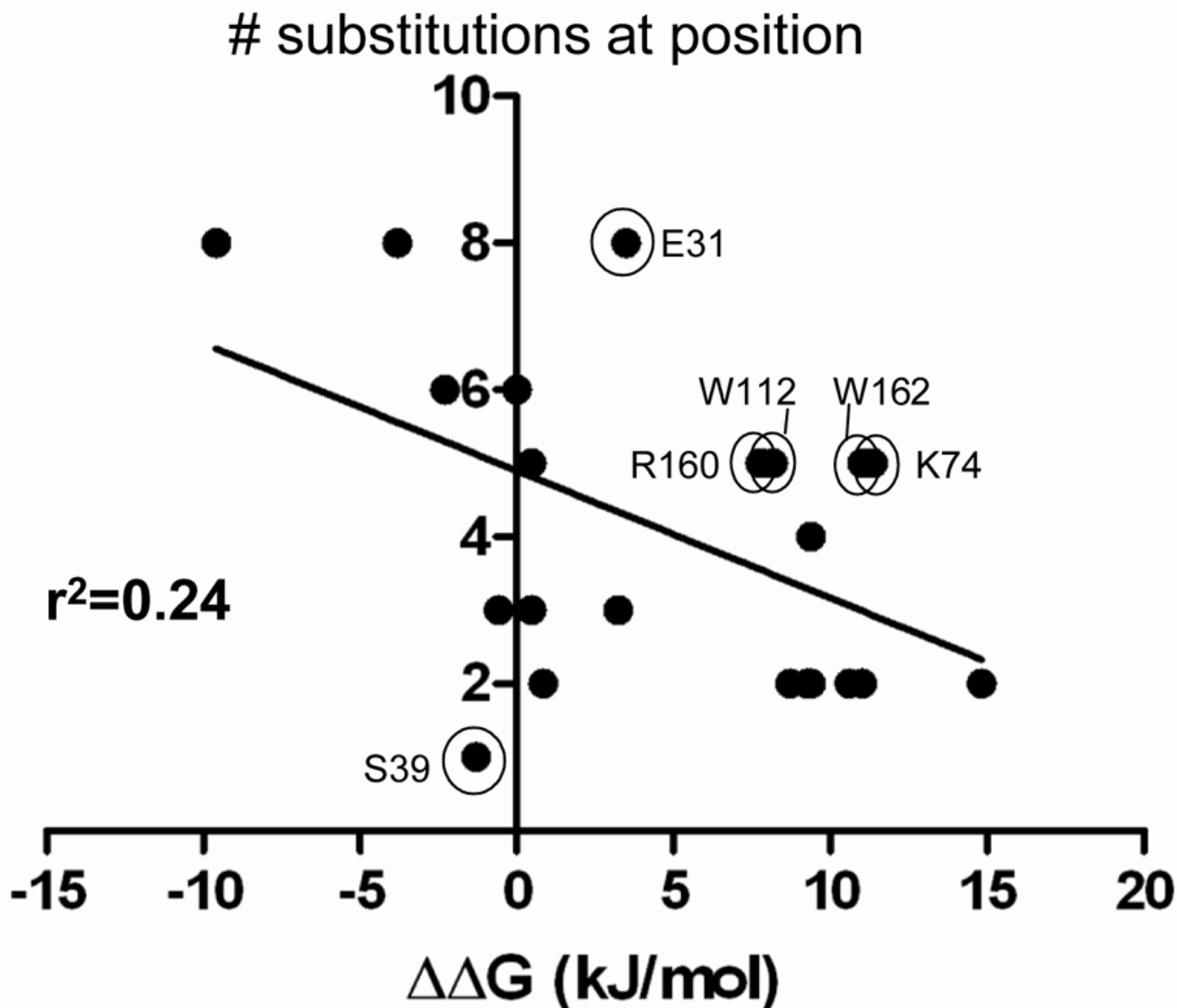


Figure 5.

The graph shows the correlation between the randomization and selection results and the $\Delta\Delta G$ values of the alanine scanning mutants at the same positions using K_i data from Zhang and Palzkill²⁸. Each data point represents one of the 23 residues randomized and shown in Fig. 3. The X-axis indicates the $\Delta\Delta G$ (kJ/mol) values for the BLIP alanine mutants for binding TEM-1 β -lactamase²⁸. The Y-axis indicates the number of amino acids (including wild type) consistent with wild type levels of BLIP function in *E. coli* TP132 (top panel sequences in Fig. 3B). The circled and labeled positions are those where there is a lack of correlation between the alanine substitution result and the randomization result. For example, the W112 position exhibits a large loss of binding affinity when mutated to alanine suggesting the position is important for binding, however, five different amino acids can reside at residue 112 and provide high level function. r^2 is the goodness of the linear regression fit of the indicated line.

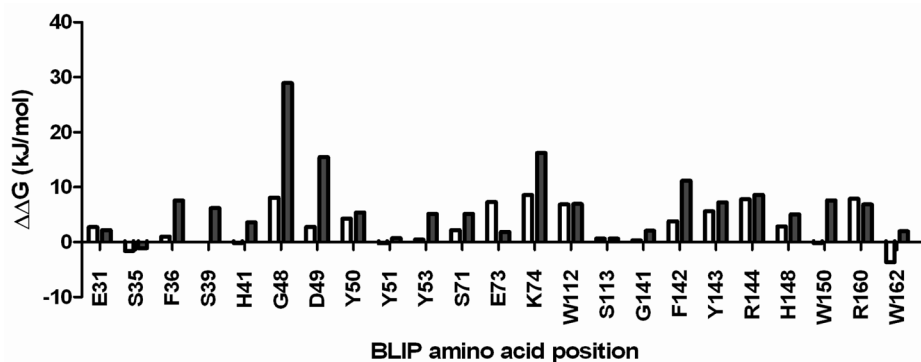


Figure 6.

The graph shows the average $\Delta\Delta G$ values for binding for BLIP mutants containing amino acid substitutions at the indicated residue positions. The X-axis indicates the BLIP residue positions that were randomized in the study. The open bar for each residue position indicates the average change in free energy of the BLIP-TEM-1 β -lactamase complex calculated using FoldX for each mutant that was found in the genetic screen to exhibit high levels of BLIP function (top panel, Fig. 3B). The value for each mutant was calculated and then the values were averaged. The filled bars indicate the average change in binding free energy calculated using FoldX for each of the 19 substitutions. The value was calculated for each of the 19 substitutions and then the values were averaged.

Table 1

Comparison of the minimum inhibitory concentration of ampicillin for growth of *E. coli* TP112 and TP132 strains containing plasmids encoding BLIP alanine-scanning mutants versus the K_i value for inhibition of TEM-1 β -lactamase by the purified BLIP derivatives. “No BLIP” refers to the *E. coli* strain containing the pTP123 plasmid that does not encode BLIP. “Wt BLIP” refers to the *E. coli* strain containing the pGR32 plasmid that encodes BLIP. The K_i data is from Zhang and Palzkill²⁸ and the $\Delta\Delta G$ values are calculated from the K_i values (Materials and Methods).

BLIP mutant	Ampicillin MIC ($\mu\text{g/ml}$)		K_i (nM)	$\Delta\Delta G$ (kJ/mol)
	<i>E. coli</i> TP112	<i>E. coli</i> TP132		
No BLIP	256	>256	-	-
Wt BLIP	1.5	4	0.5	-
E31A	8	12	2.0	3.4
S35A	1.5	4	0.5	0
F36A	24	24	40	10.8
S39A	1.5	4	0.3	-1.3
H41A	64	64	34	10.4
G48A	2	12	0.7	0.8
D49A	3	16	20	9.1
Y50A	1	4	0.01	-9.7
Y51A	1.5	4	0.5	0
Y53A	4	24	21	9.3
S71A	3	6	0.2	-2.3
E73A	1.5	6	0.4	-0.5
K74A	3	24	46	11.2
W112A	1.5	16	13	8.0
S113A	1	4	0.1	-4.0
G141A	3	8	1.8	3.18
F142A	2	16	16	8.58
Y143A	1	4	0.6	0.5
R144A	1	6	0.6	0.5
H148A	24	32	21	9.3
W150A	96	256	184	14.6
R160A	2	24	11	7.7

Table 2

Comparison of the minimum inhibitory concentration of ampicillin for growth of *E. coli* TP112 and TP132 strains containing plasmids encoding the various BLIP mutants versus the K_i value for inhibition of TEM-1 β -lactamase by the purified BLIP derivatives.

BLIP mutant	Ampicillin MIC ($\mu\text{g/ml}$)		K_i (nM)
	<i>E. coli</i> TP112	<i>E. coli</i> TP132	
No BLIP	256	>256	-
Wt BLIP	1.5	4	0.5
E31Q	1.5	6	1.4
D49S	2	32	16
E73Y	1.5	12	3.7
W112R	1.5	12	3.9
F142G	1.5	16	12