Published in final edited form as:

J Mol Biol. 2011 March 11; 406(5): 730–744. doi:10.1016/j.jmb.2011.01.014.

Identification of a β -lactamase inhibitory protein variant that is a potent inhibitor of *Staphylococcus* PC1 β -lactamase

Ji Yuan^{1,2}, Dar-Chone Chow^{1,2}, Wanzhi Huang², and Timothy Palzkill^{2,3,*}

²Department of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 USA

³Department of Biochemistry and Molecular Biology Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 USA

Abstract

The β -lactamase inhibitory protein (BLIP) binds and inhibits a diverse collection of class A β -lactamases. Widespread resistance to β -lactam antibiotics currently limits treatment strategies for *Staphylococcus* infections. The goal of this study was to determine the binding affinity of BLIP for *S. aureus* PC1 β -lactamase and to identify mutants that alter binding affinity. The BLIP inhibition constant (K_i) for the PC1 β -lactamase was measured at 350 nM and isothermal titration calorimetry (ITC) experiments indicated a binding constant (K_d) of 380 nM. A total of 23 residue positions in BLIP that contact β -lactamase were randomized and phage display was used to sort the libraries for tight binders to immobilized PC1 β -lactamase. The BLIP K74G mutant was the dominant clone selected and it was found to inhibit the PC1 β -lactamase with a K_i of 42 nM while calorimetry indicated a K_d of 26 nM. Molecular modeling studies suggested BLIP binds weakly to the PC1 β -lactamase due to the presence of alanine at position 104 of PC1. This position is occupied by glutamate in the TEM-1 enzyme where it forms a salt bridge with BLIP residue Lys74 that is important for the stability of the complex. This hypothesis was confirmed by showing that the A104E PC1 enzyme binds BLIP with 15-fold greater affinity than wild type PC1 β -lactamase. Kinetic measurements indicated similar association rates for all complexes with the variation in affinity due to altered dissociation rate constants suggesting changes in short-range interactions are responsible for the altered binding properties of the mutants.

Keywords

antibiotic resistance; phage display; protein-protein interactions; salt bridge; enzyme inhibitors

Introduction

The β -lactamase inhibitory protein (BLIP) binds and inhibits a wide range of class A β -lactamases from a diverse set of Gram-positive and -negative bacteria 1, 2. BLIP is a 165 amino acid protein and is produced by the soil bacterium *Streptomyces clavuligerus* 3. This organism also produces the small molecule, mechanism-based inhibitor of β -lactamase

* To whom correspondence should be addressed: timothy.palzkill@bcm.edu, Phone: 713-798-5609; Fax: 713-798-7375;.

¹These authors contributed equally to the work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

named clavulanic acid 4. Gene knockout studies indicate BLIP is not essential for growth and the exact role of BLIP in the biology of *S. clavuligerus* remains unknown 4.

β -lactamases catalyze the hydrolysis of β -lactam antibiotics to provide bacteria with resistance to these drugs. They are grouped into four classes (A, B, C and D) according to amino acid sequence homology 5. Enzymes from classes A, C and D are serine hydrolases while class B enzymes are zinc metallo-enzymes unrelated in structure to the other classes 5. Class A β -lactamases exhibit broad substrate hydrolysis profiles which include penicillins, cephalosporins and, for a few enzymes, carbapenems. TEM-1 β -lactamase is a class A enzyme and is a common plasmid-encoded β -lactamase in Gram-negative bacteria 5. Numerous other class A β -lactamases such as SHV-1, SME-1, Bla1 and PC1 have substrate profiles similar to TEM-1 and are found in various Gram-positive and Gram-negative bacteria 5.

The interaction between BLIP and class A β -lactamases has become a model system for studying the amino acid sequence determinants of binding energy in a protein-protein complex 2; 6; 7; 8; 9; 10; 11; 12. BLIP inhibits class A β -lactamases with a wide range of affinities. For example, it inhibits TEM-1 β -lactamase with a K_i of 0.5 nM but inhibits the SHV-1 β -lactamase, which is 68% identical in amino acid sequence to TEM-1, with a K_i of 1.1 μ M 13. High-resolution co-crystal structures of BLIP in complex with TEM-1 and SHV-1 β -lactamase are available and suggest a subtle difference between TEM-1 and SHV-1 (TEM-1 Glu104 versus SHV-1 Asp104) is responsible for the large difference in affinity 9; 14. The BLIP- β -lactamase interface has been extensively studied using structural, computational and biochemical approaches 2; 6; 9; 12; 14. In addition, 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 2; 12; 13. 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 2; 12.

The goal of this study was to determine the binding affinity of BLIP for *Staphylococcus aureus* PC1 β -lactamase 15, and to identify mutants that alter binding affinity in order gain insights into the determinants of binding specificity in protein interactions as well as to generate a potent inhibitor of the enzyme.

S. aureus is an important human pathogen and penicillin had been a drug of choice for treatment of infections caused by this organism. Currently, however, a large percentage of *S. aureus* human infections are mediated by penicillin-resistant bacteria 16. Two mechanisms are responsible for penicillin resistance in *S. aureus*. One mechanism involves the acquisition of a new penicillin binding protein, PBP2a, to create methicillin resistant *S. aureus* strains (MRSA) which are not inhibited by most β -lactam antibiotics 17. MRSA have become widespread in both the hospital and community setting and by 2003 greater than 50% of *S. aureus* strains isolated in hospitals were MRSA 18. A second mechanism involves the production of β -lactamases, encoded by the *blaZ* gene, which inactivate penicillin by catalyzing the hydrolysis of the β -lactam ring 16. The β -lactamase mediated mechanism of resistance is associated with the *blaZ* gene as well as its transcriptional repressor gene, *blaI*, and a gene encoding a sensor transducer protein, *blaR1* 19. Many MRSA strains also encode β -lactamase and so the development of inhibitors of the *S. aureus* β -lactamase and PBP2a enzymes is an important goal for improved antibiotic therapy. In this regard, a BLIP-based *S. aureus* β -lactamase inhibitor could be useful as a potential therapeutic or diagnostic reagent.

The *blaZ* encoded enzymes are β -lactamases that have been categorized to four types (A, B, C, D) based on serotyping and substrate specificity 20. The A, C, and D types are commonly encoded on plasmids while *blaZ* gene for type B enzymes is often present on the chromosome 21; 22. Despite the separation into distinct types, the *S. aureus blaZ* encoded enzymes are very similar; with >90% amino acid sequence identity across the various types 23. The PC1 β -lactamase is a type A, plasmid encoded enzyme that provides for antibiotic resistance by catalyzing the hydrolysis of β -lactams such as penicillin G and ampicillin 24; 25; 26; 27.

Previously, we used phage display to identify substitutions in the loop regions of BLIP that insert into the TEM-1 β -lactamase active site to identify tight binding variants 28. In this study, a set of 23 BLIP libraries, each of which is randomized for a single BLIP residue that is hypothesized to be in contact with PC1 enzyme based on other BLIP β -lactamase X-ray structures, were screened using phage display to identify variants that bind tightly to the *S. aureus* PC1 β -lactamase (Fig. 1 A,B). A single variant, K74G, predominated among the clones selected by phage display and subsequent purification and binding characterization indicated this BLIP mutant is a potent inhibitor of the PC1 β -lactamase. In addition, molecular modeling suggested the presence of alanine at position 104 in the PC1 enzyme rather than glutamate, which is present in TEM-1 β -lactamase, is responsible for the weak binding of PC1 to wild type BLIP. This hypothesis was confirmed by demonstrating that the A104E PC1 β -lactamase binds BLIP 15-fold tighter than wild type PC1. Furthermore, kinetic analysis showed that the differences in the affinity of the complexes are mainly due to the differences in dissociation rate constants. These findings suggest that alterations in short range interactions, such as unbalanced charges, are responsible for the observed difference in binding affinity of the BLIP and PC1 β -lactamase proteins.

Results

Phage display of BLIP combinatorial libraries

The *blaZ*-encoded PC1 β -lactamase is a common source of penicillin resistance in *S. aureus* 19. BLIP has previously been shown to bind and inhibit β -lactamases from Gram-positive and Gram-negative bacteria with a wide range of affinities 1; 2. It was therefore of interest to determine the inhibition constant of wild type BLIP for the PC1 β -lactamase. For this purpose, recombinant PC1 enzyme was expressed and purified from *E. coli* (Experimental Procedures) 27; 29. BLIP was found to inhibit the PC1 β -lactamase with a K_i of 350 nM (Experimental Procedures). This level of inhibition is approximately 700-fold less potent than that observed for BLIP inhibition of TEM-1 but is roughly 2-fold more potent than inhibition of SHV-1 β -lactamase 2; 9; 13.

In order to explore the determinants of binding specificity for the BLIP-PC1 β -lactamase interaction and to identify variants of BLIP with increased potency, phage display was used to screen combinatorial libraries for variants that bind tightly to the PC1 enzyme (Fig. 2). A phage display vector was developed for the efficient display and enrichment of BLIP variants. The pTP378 plasmid used for BLIP display utilizes the AraC-P-bad arabinose promoter regulation system to tightly control the expression of the BLIP-bacteriophage gene III protein fusion by the presence or absence of arabinose (Fig. 2) 30; 31. In addition, a trypsin cleavage site was engineered between BLIP and the gene III protein to allow elution of phage particles from the solid phase by digestion with trypsin 32; 33.

A total of 23 BLIP combinatorial libraries, each randomized at a single codon position in BLIP, were used for the phage display panning (Fig. 1). As described above, alanine scanning mutagenesis had previously been performed at 23 BLIP positions that, based on the X-ray structure, are in contact with TEM-1 β -lactamase in the protein complex to

determine the positions important for binding affinity and specificity [2, 12]. These 23 positions were randomized individually in a recent study using a genetic screen to study BLIP binding to TEM-1 β -lactamase [11]. The previously constructed libraries were transferred to the pTP378 vector and the resultant 23 phage libraries were pooled and used for biopanning on PC1 β -lactamase that had been immobilized to the solid phase (Experimental Procedures). DNA sequencing of clones from the pool of libraries indicated diversity among the clones with no strong bias towards individual mutants (Supplementary Table 1). Two rounds of biopanning enrichment were performed and individual clones were evaluated for binding after each round using a 96-well phage ELISA procedure (Experimental Procedures) [34]. Those phage clones that exhibited ELISA signals greater than or equal to those obtained for binding to immobilized PC1 β -lactamase by the control phage displaying the wild type BLIP protein were subjected to DNA sequencing.

The DNA sequencing results indicated that the pooled libraries converged on the K74G variant as it became the dominant clone after biopanning (Table 1). It is of interest to note that among the K74G mutants selected, multiple different glycine codons are represented, which indicates that unique K74G mutants were independently selected rather than a single clone dominating the population (Table 1). This, in turn, suggests, first, that the library has high sequence coverage, in that it contained clones representing all codons for glycine and, second, that many mutants were sampled but that K74G exhibits the optimal properties for enrichment over other variants in the biopanning experiment.

Biochemical characterization of BLIP K74G

The fact that the BLIP K74G variant dominates the population of phage-selected mutants suggests that it binds PC1 β -lactamase tighter than wild type BLIP or other mutants in the library. This was tested by introducing the K74G substitution into BLIP in a protein expression plasmid by site directed mutagenesis and purification of the recombinant protein. The inhibition constant of the purified K74G BLIP variant for PC1 β -lactamase was found to be 42 nM indicating an approximately 10-fold increase in potency relative to wild type BLIP (Table 2, Fig. 3). The increased potency of the K74G variant relative to wild type is consistent with the finding that this mutant becomes the dominant clone in the phage display binding competition.

The binding properties of the wild type BLIP and the K74G mutant with PC1 β -lactamase were investigated using isothermal titration calorimetry (ITC) (Table 2, Fig. 4). Consistent with the findings of the inhibition assays, ITC experiments indicated wild type BLIP interacted with PC1 β -lactamase with a binding constant (K_d) of 380 nM. Similarly, the K74G BLIP protein was found by calorimetry to bind with a K_d of 26 nM, which is similar to the inhibition constant of 42 nM (Table 2, Fig. 4). Therefore, the inhibition constant is a good reflection of the physical binding affinity and the results further confirm that a single amino acid substitution results in an approximately 10-fold increase in binding affinity between BLIP and the PC1 enzyme.

BLIP K74G binding to TEM-1 β -lactamase

It was also of interest to determine the impact of the K74G substitution on the binding and inhibition of the TEM-1 β -lactamase. The results of the inhibition assay indicated that BLIP K74G exhibits a K_i of 59 nM for TEM-1, which reflects an approximately 100-fold loss in potency relative to inhibition by wild type BLIP. This result is consistent with the previous finding that the K74A BLIP mutant displays a K_i of 46 nM and that Lys74 is an important determinant of binding affinity between BLIP and TEM-1 β -lactamase [2, 12]. Therefore, the K74G BLIP variant exhibits a large change in binding specificity in that inhibition of TEM-1 by wild type BLIP ($K_i = 0.5$ nM) is 700-fold more potent than inhibition of PC1 (K_i

= 350 nM) while K74G BLIP inhibits both TEM-1 (K_i = 59 nM) and PC1 β -lactamases (K_i = 42 nM) with similar potency.

A104E PC1 β -lactamase binding to BLIP

The X-ray structure of the complex between TEM-1 β -lactamase and BLIP indicates a buried salt bridge is formed between Lys74 of BLIP and Glu104 of TEM-1 14. The structure of the PC1 β -lactamase complex with BLIP has not been solved, however docking of the PC1 structure into the analogous position as TEM-1 in the BLIP complex indicates that alanine at position 104 of PC1 precludes formation of the salt bridge and modeling suggests a A104E substitution in PC1 would allow a salt bridge to be formed which could result in tighter binding between the proteins (Fig. 5). This hypothesis was tested by constructing the A104E PC1 β -lactamase mutant, purifying the enzyme, and testing binding by inhibition and calorimetry assays (Table 2). Consistent with the hypothesis, it was found that BLIP inhibits the A104E PC1 enzyme with a K_i of 24 nM, which is 15-fold more potent than inhibition of wild type PC1. In addition, ITC experiments indicated a K_d of 20 nM demonstrating the inhibition and binding constants are similar and suggesting that glutamate at position 104 allows the formation of a salt bridge with Lys74 of BLIP.

Molecular modeling of BLIP with PC1 β -lactamase also suggests that the BLIP K74G mutant would exhibit reduced binding affinity for the A104E PC1 enzyme since the potential for a salt bridge interaction would be lost. Consistent with this prediction, the BLIP K74G inhibits the PC1 A104E enzyme with a K_i of 480 nM and ITC experiments indicate a K_d of 900 nM for the interaction (Table 2). Taken together, the results of the BLIP K74G and PC1 A104E experiments suggest the ability of positions 74 in BLIP and 104 in β -lactamase to form a salt bridge is an important determinant of binding specificity. However, taken together, the results indicate that it is not a special feature of a salt bridge that is necessary for potent binding but rather it is critically important to not have unbalanced charges buried in the protein-protein interface. More generally, the results indicate it is possible to manipulate the specificity of protein-protein interactions by the addition and subtraction of charged residues that are buried in the complex interface.

Biochemical characterization of BLIP K74A

As seen in Table 1, the BLIP K74G is clearly the dominant clone selected after two rounds of phage display panning. The fact that glycine dominates suggests other substitutions at position 74 are less potent. This hypothesis was tested by examining the potency of the BLIP K74A mutant for inhibition of PC1 β -lactamase. It was found that BLIP K74A has a K_i of 129 nM for PC1 by the inhibition assay and a K_d of 71 nM determined by ITC (Table 2). Therefore, BLIP K74A is a more potent inhibitor of PC1 β -lactamase than wild type BLIP but is less potent than K74G, which is consistent with finding that K74G is the dominant clone after panning. In addition, it was found that BLIP K74A follows the same trend as K74G in that it exhibits reduced inhibition of the PC1 A104E β -lactamase relative to wild type BLIP (Table 2).

Kinetic characterization of binding interactions

It has been shown that long-range electrostatic interactions can increase association rates which, in turn, increase affinity 35, 36. Therefore it is possible that altering the charge of residues at 74 in BLIP and 104 in β -lactamase alters the association rate of the complexes. The kinetics of association and dissociation of the wild type and mutant complexes were therefore determined to gain insights into the mechanism by which the K74G mutations alters affinity (Fig. 6). It was found that all of the complexes, regardless of charge status, exhibited similar association rate constants in the condition of 50 mM phosphate, pH 7.0, 150 mM NaCl (Fig. 6A and Table 3). In contrast, the dissociation rate constants of these

complexes exhibited significant differences and are largely responsible for the observed differences in the affinities of the complexes (Table 3). The calculated K_d values based on the kinetic rate constants (Table 3) are about 2~3 fold smaller than the K_i and K_d (Table 2) from the inhibition assay and ITC measurements. These variations may be due to the differences in the experimental conditions. Dissociation processes are influenced by short-range interactions such as hydrogen bonds and van der Waals interactions and the results suggest the unbalanced charge in the PC1 wt/BLIP K74 and PC1 A104E/BLIP K74G complexes disrupts these interactions leading to faster dissociation rates.

Discussion

Phage display and biochemical characterization experiments in this study revealed the BLIP K74G mutant as a tight binding inhibitor of the PC1 enzyme ($K_i = 42$ nM, $K_d = 26$ nM). Position 74 had previously been shown to be an important determinant of binding specificity for the BLIP interaction with the TEM-1 and SME-1 β -lactamases 2³ 12. In the case of TEM-1 inhibition, substitution of Lys74 with alanine results in a nearly 100-fold loss in potency for BLIP. In contrast, the K74A BLIP variant exhibits an approximately 20-fold increased potency for inhibition of SME-1 β -lactamase 2. Similarly, as shown here, the K74G BLIP variant exhibits 10-fold increased inhibition of PC1 β -lactamase but displays approximately 100-fold reduced inhibition of TEM-1 β -lactamase. Clearly, the effect of substitutions at position 74 is context dependent, i.e., the impact of the mutation depends on the identity of the β -lactamase binding partner. These findings further establish the important role of position 74 in controlling the specificity of BLIP binding to class A β -lactamases and points to charged residues in protein-protein interaction interfaces as key determinants of binding specificity.

Molecular modeling of PC1 β -lactamase docked into a similar position as TEM-1 in a BLIP- β -lactamase complex suggests that substitution of Ala104 in PC1 could create a salt bridge with Lys74 of BLIP and potentially increase binding affinity. This prediction was confirmed by creating the A104E PC1 enzyme and showing by an inhibition assay and calorimetry that it binds BLIP approximately 15-fold tighter than does wild type PC1 β -lactamase. It is of interest in this regard that position 104 in SHV-1 β -lactamase has also been shown to be an important determinant of binding affinity with BLIP 9. BLIP inhibits SHV-1 β -lactamase weakly with a K_i of approximately 1 μ M which is surprising in that TEM-1 is inhibited at a K_i of 0.5 nM and SHV-1 is 68% identical in amino acid sequence to TEM-1 9³ 13. TEM-1 encodes Glu104 while SHV-1 contains Asp104 and it was hypothesized based on X-ray structures of TEM-1 and SHV-1 with BLIP that the reduced volume of Asp104 disrupts the salt bridge with BLIP Lys74 and leads to an electrostatic clash with BLIP Glu73 9 (Fig. 1C). Strong support for this hypothesis was provided by the demonstration that the SHV-1 D104E enzyme was inhibited with approximately 1000-fold more potency than wild type SHV-1 by BLIP with a K_i of 1.2 nM 9. The previous mutagenesis results with SHV-1 position 104 and those presented here for PC1 clearly indicate this position plays a key role in determining the binding specificity of BLIP for class A β -lactamases.

The importance of charge matching between residue 74 of BLIP and residue 104 of PC1 β -lactamase can be dissected using the thermodynamic cycle shown in Figure 7. The results show that substitutions at BLIP K74 and PC1 A104 are strongly coupled, i.e., the effect on binding of a substitution at these positions depends on the identity of the other residue. If residues act independently, the change in binding energy of the double mutant (designated below as X,Y) relative to wild type should equal the sum of the change in binding energy of each single mutant (X and Y) as indicated in equation 1 37³ 38. The ΔG_I term is

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} + \Delta G_I \quad (\text{eq. 1})$$

the coupling energy that reflects the extent to which the change in interaction between X and Y influences the binding energy. For residue positions that act independently, ΔG_I is zero. In the case of the PC1 A104E/BLIP K74G double mutant cycle, ΔG_I is very large at +3.9 kcal/mol indicating strong unfavorable coupling between PC1 residue 104 and BLIP residue 74. The unfavorable coupling energy is due to the large penalty in binding energy for having an unmatched charged residue in the interface (Fig. 7). A similar thermodynamic cycle analysis can be performed for the PC1 A104E/BLIP K74A mutant and G_I is also large at +3.1 kcal/mol, further indicating the importance of charge matching between PC1 residue 104 and BLIP residue 74 (Fig. 7B).

As indicated above, a simple model in which the TEM-1/BLIP structure (1JTG) is substituted with an aligned PC1 structure (3BLM), shows that the PC1 Glu104 and BLIP Lys74 residues can be accommodated (Fig. 5). The question of why glycine at position 74 of BLIP is the best substitution for the tight binding to PC1 β -lactamase is more difficult to discern. The loss of the lysine side chain in BLIP coupled with the presence of alanine at 104 in PC1 could generate a relatively large cavity in the interface (Fig. 5) and it is thought that a cavity within an interface is deleterious to the stability 39. Nevertheless, BLIP K74G binds tightly to wild type PC1 which may indicate the glycine residue allows a structural rearrangement in the region to remove the cavity. This possibility is supported by the finding that BLIP K74A, which would create less of a cavity, binds more weakly to PC1 than does BLIP K74G (Table 2).

Many observations have shown that salt bridges play significant roles in the specificity of binding, however, the contribution of charge-charge electrostatic interactions to binding energetics appears to be minimal 40; 41; 42; 43; 44. For example, when Arg43 of human growth hormone receptor was changed to Leu to eliminate the salt bridge between Arg43 of the receptor and Asp171 of hGH while maintaining van der Waals interactions from the bulky alkyl component, the K_d for hGH only doubles while the mutant receptor acquires a new binding cross-reactivity with bovine growth hormone 41. In the case of the HyHEL-10 monoclonal antibody and hen egg-white lysozyme antigen interaction complex, the HyHEL-10 antibody forms a salt bridge from Asp32 of the heavy chain to Lys97 of hen egg-white lysozyme antigen 44. The interaction between the D32N heavy chain mutant of HyHEL-10 and K97M mutant of HEWL (which eliminates the salt bridge while maintaining most van der Waals interactions), however, exhibits only small changes in affinity 44. These observations suggest that salt bridges can be replaced with nonelectrostatic interactions with little effect on the binding strength 44. Compared to these two systems, the interfaces of PC1 A104E/BLIP K74 and PC1 A104/BLIP K74G are significantly different, especially in terms of the potential cavity generated in the later complex. The consistent feature, however, is that the charge-charge electrostatic interactions have almost no effect on the overall binding energetics unless there is an unsatisfied charge in the interface (Fig. 7).

The ITC measurements provide information on the thermodynamics of the interactions. At 30 °C, all the binding interactions have favorable enthalpy driving forces. The tight complexes (PC1 A104/BLIP K74G and PC1 A104E/BLIP K74) have strong enthalpy driving forces, suggesting that these complexes achieve their binding strength through many well formed bonds, such as salt bridges, hydrogen bonds, and van der Waals interactions. The weak complex PC1 A104/BLIP K74 (wt/wt) has the lowest enthalpy driving force which is compensated by a larger entropy driving force. Unexpectedly, the weak complex of

PC1 A104E/BLIP K74G exhibits the strongest enthalpy driving force, but an unfavorable entropy driving force.

The binding thermodynamics reflect general changes of the entire system and not just the site of the substitution, and therefore reflect the global and coupling effects of the substitution. One can assess the energetic coupling of a contact by the deviation of the expected binding thermodynamics due to a substitution. The case of the binding thermodynamics of PC1 A104E/BLIP K74G interaction is a good example. The pairwise direction interaction between the Glu residue of A104E PC1 and the Gly of K74G BLIP is not a strong bond, therefore is expected to have a weaker enthalpy driving force (less negative binding enthalpy). Table 2 shows, however, that this binding enthalpy is the strongest among the four complexes (more favorable than the tight complexes of PC1 A104/BLIPK74G and PC1 A104E/BLIPK74). The strongly favorable binding enthalpy suggests that adjustments of other interactions in the interface may lead to stronger bond interactions between PC1 A104E and BLIP K74G proteins, at the expense of the binding entropy. The sources of the unfavorable binding entropy could be that the proteins are more flexible in the complex than the unbound forms, or the complex immobilizes more water molecules than the unbound form, or a combination of these effects.

In contrast, the other weak complex (PC1 A104/BLIPK74) exhibits quite different binding thermodynamics with a binding enthalpy significantly reduced relative to the tight binding complexes, which is predicted based on the expected weak bond between Ala104 of PC1 and Lys74 of BLIP. A favorable binding entropy for this complex suggests that desolvation of the interface may play an important role in driving the binding reaction

Experimental Procedures

Protein expression and purification

Bacteria containing the PC1 β -lactamase expression plasmid pTS32 29 were grown in LB media at 37 °C to $A_{600\text{ nm}}$ of ~ 1.2 , and the culture was induced with 2 mM IPTG and grown at room temperature for 6 hours. The bacteria were harvested by centrifugation at 5000 rpm for 10 minutes at 6 °C. The bacterial pellets were resuspended in 50 mM phosphate, pH 7.4 and the bacteria were lysed using a French press and sonication. The bacterial lysate was clarified by centrifugation 100,000 g for 2 hr. The clarified lysate was passed through an SP ion exchange column. The bound protein was eluted using a step (2.5 M NaCl in 50 mM phosphate, pH 7.4) elution. The eluted protein was further purified using a S75 sizing chromatography step in PBS buffer (50 mM phosphate pH 7.0, 150 mM NaCl). The wild type and K74G mutant BLIP proteins were expressed and purified as previously described [11, 45]. Briefly, *E. coli* bacteria (strain RB791) containing BLIP mutant expression plasmid (pGR32 13) were grown in LB media at 37 °C until the $A_{600\text{ nm}}$ was ~ 1.2 . The culture was induced with 2 g/L of lactose at room temperature for 6 hours and harvested by centrifugation at 5000 rpm for 10 minutes at 6 °C. The bacterial pellets were resuspended in B-PER Bacterial Protein Extraction solution (Pierce, Rockford, IL) or a prepared equivalent (1% Triton-X 100 in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) at a ratio of 15 ml for 1 g of bacterial pellet. The resuspensions were vigorously shaken for 20 min at room temperature, and then centrifuged at high speed (15000 rpm in a Beckman type 35 rotor) for 30 min. The supernatants were mixed by stirring with Talon cobalt resin overnight. The Talon cobalt resin was allowed to settle by gravity and then collected and washed three times. The bound BLIP mutant proteins were eluted from the Talon cobalt resins using 150 mM imidazole in TBS. The eluted proteins were further purified with S75 sizing chromatography in PBS buffer (50 mM phosphate pH 7.0, 150 mM NaCl). The purity of the proteins was confirmed by SDS-PAGE analysis. Protein concentrations were determined using optical density

measurements at 280 nm based on the theoretically calculated extinction coefficients according to the amino acid sequence.

Isothermal titration calorimetric measurements

The binding enthalpy measurements were carried out using a VP-ITC isothermal titration calorimeter from MicroCal LLC (Northampton, MA). Titration experiments were carried out either by titrating a BLIP protein (30 ~ 70 μ M of wild type or K74G mutant) into PC1 β -lactamase (4 ~ 6 μ M) or by titrating A104E PC1 β -lactamase mutant protein (166 μ M) into wild type or K74G mutant proteins (6 ~ 9 μ M) in PBS buffer (50 mM phosphate pH 7.0, 150 mM NaCl). ITC experiments of each BLIP protein binding to PC1 β -lactamase were done at no less than three different temperatures within the range of 6 °C to 30 °C. The data were processed using the program Origin 7.0 with the manufacturer provided ITC processing add-on module.

Phage display of BLIP random libraries

A phage display vector, pTP378, was constructed containing the BLIP fused at its C-terminus to the M13 bacteriophage gene III protein. In addition, a trypsin cleavage site was placed between β -lactamase and the gene III protein 33. The BLIP-gene III protein fusion is under the transcriptional control of the *E. coli* AraC transcription factor and its expression is induced by the addition of arabinose to the growth medium 30. This plasmid is based on the pTP179 plasmid that was previously shown to exhibit tightly controlled expression of the β -lactamase-gene III fusion protein in response to the presence or absence of arabinose 31.

Each BLIP library was randomized for a single codon to the sequence NNN where N is any of the 4 nucleotides. A total of 23 BLIP random libraries were previously constructed in the pGR32 plasmid 11. Each of these libraries was transferred from the pGR32 plasmid to pTP378 by PCR amplification from the pGR32 library, digestion with the NdeI and XbaI restriction endonucleases, and insertion into the pTP378 plasmid that was digested with the NdeI and XbaI. This replaced the BLIP gene in pTP378 with the BLIP gene that is randomized at a given codon. The BLIP mutant-gene III protein fusions are therefore under the transcriptional control of the *E. coli* AraC transcription factor and expression is induced by the addition of arabinose to the growth medium 30. In addition, the presence of the trypsin cut site between BLIP and gene III protein is available for elution of phage from the target 32, 33. The DNA sequence of several individual clones from each library was determined to ensure the structure of each library was correct. After each of the 23 BLIP libraries had been transferred to the pTP378 phage display vector, the libraries were pooled to create a single library that was used to screen for mutants with altered binding properties. A total of 25 clones were picked from the pooled library to check diversity and no obvious bias in sequences occurred. The wild type gene was detected 3 times and W150I was detected twice while the remaining 20 clones all had different sequences (Supplementary Table 1).

The phage display biopanning was performed by coating purified *S. aureus* PC1 β -lactamase into an immunotube at a concentration of 50 μ g/ml in PBS, pH 7.2, at 4°C with an overnight incubation. The immunotube was washed 3 $\times$ with PBS and blocked with 2% milk in 1 $\times$ PBS for 2 hours at room temperature. After washing three times with PBS, 1.6×10^{13} phages from the 23 pooled BLIP random libraries were allowed to bind to the immobilized PC1 β -lactamase in PBS with 2% milk for two hours at room temperature. Unbound phages were removed by washing the tube 20 times with PBS with 0.1% Tween 20. Bound phages were eluted by the addition of 1 mg/ml trypsin at room temperature for 20 minutes which releases bound phages by cleaving between BLIP and gene III protein. A small aliquot of the eluted phages was used to determine the titer of the elution by infection of *E. coli* TG1 and

spreading the cells on LB agar plates containing 12.5 mg/ml chloramphenicol. The remainder of the eluted phages was used to infect *E. coli* TG1 to amplify the phage stock for subsequent rounds of biopanning. A second round of biopanning was performed using identical conditions but starting with 2.7×10^{13} input phages.

Single point phage ELISA was used to screen clones after each round of panning 34. For this purpose, individual clones were picked after each round of panning and inoculated into wells of a 96-well 2 ml deep-well plate containing 1.2 ml of 2YT growth medium with 12.5 µg/ml chloramphenicol and 8×10^9 KM13 helper phages for the production of phage particles from the pTP378 phagemid-based vector. The deep well plate was incubated overnight with shaking and then centrifuged to pellet the *E. coli* cells. The supernatants were the phage preparations that were used for ELISA. Purified PC1 β-lactamase was coated into wells of a microtiter plate in PBS at a concentration 50 µg/ml. The wells were blocked with 2% milk in PBS for two hours at room temperature, washed with PBS, and 200 µl of each phage preparation was added and allowed to bind. After washing 3× with PBS with 0.025% Tween 20, bound phages were detected with an anti-M13 antibody conjugated with horseradish peroxidase and ABTS substrate. Those clones that resulted in an ELISA signal greater than or equal to the wild type BLIP control were subjected to DNA sequencing to determine the identity of the mutant.

Determination of inhibition constants

The inhibition constant for the *S. aureus* PC1 β-lactamase inhibition by BLIP was measured by monitoring β-lactam hydrolysis in the presence of increasing concentrations of BLIP. The inhibition assays were performed as described previously with minor modifications 2: 13. Briefly, for tight binding, 10 nM of PC1 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 1 µM and the initial velocity of the reaction was monitored at 482 nm. For weak binding, 500 nM of PC1 β-lactamase was used and 100 µM cephalosporin C was used as the substrate and the initial velocity of hydrolysis was monitored at 280 nm. The initial velocity of substrate hydrolysis was plotted versus BLIP concentration and the resulting curve was fit to the following equation for a tight binding inhibitor to obtain the inhibition constant K_i as described previously.

$$E_{free} = \frac{-(K_i + I_{total} - E_{total}) + \sqrt{(K_i + I_{total} - E_{total})^2 + 4(E_{total} \times K_i)}}{2} \quad (1)$$

Where E_{free} is the active enzyme determined by the measured initial velocity. E_{total} , I_{total} and K_i are the total concentration of enzyme and inhibitor and the inhibition constant, respectively.

Modeling of the BLIP-PC1 β-lactamase complex

The model was constructed using Swiss pdb viewer by aligning the PC1 β-lactamase structure (3BLM) with TEM-1 of the BLIP/TEM-1 structure (chain A of 1JTG) and merging the aligned PC1 β-lactamase with BLIP (chain B of 1JTG) 14. The mutation at position 104 of PC1 β-lactamase was adjusted using the Coot program to identify the rotamer consistent with a salt bridge interaction 46.

Kinetic measurements of the association and dissociation rates of protein complexes

The association rate constants of the binding of BLIP and PC1 β -lactamase proteins were all determined using a stopped-flow fluorescence spectrometer as previously described 10. In addition, the association rate constants of the two tight binding complexes (PC1 A104E/BLIP K74 and PC1 A104/BLIP K74G) were also determined using an enzymatic activity-based measurement. These two methods cross validate each other. The stopped-flow fluorescence spectrometry measurements were carried out using a SLM 48000 fluorescence spectrometer equipped with a MilliFlow stopped-flow rapid mixing reactor accessory. The intrinsic fluorescence (283 nm excitation wavelength and emission wavelength 310 nm) of the binding proteins was monitored with 5 μ M of the various BLIP and PC1 β -lactamase proteins loaded into the injection syringes for rapid mixing. For the activity-based association measurements of tight binding complexes, 20 nM of PC1 β -lactamase was mixed with an equal volume of 50 nM of BLIP proteins. Nitrocefin hydrolysis activity of the mixture was determined at designated time points after mixing. The nitrocefin hydrolysis activity was relatively constant within a short period (<10 sec) and treated as instantaneous activity. This instantaneous activity of the mixed sample decreased as mixing time increased due to inhibition resulting from BLIP binding. The amount of non-inhibited, active enzyme was calculated from the instantaneous activity and treated as a function of time, which was fitted to the kinetic equation accounting for the second order association and first order dissociation (equation 2).

Kinetic measurements of the dissociation rate constants of protein complexes

For dissociation measurements, 10 μ M of the complex was diluted 100 to 400-fold into a solution containing nitrocefin, and nitrocefin hydrolysis was continuously monitored by optical absorbance at 482 nm. The nitrocefin hydrolysis rates were converted to active enzyme concentrations using an extinction coefficient of 15900 M⁻¹cm⁻¹ and k_{cat} was determined for that condition. The K_M values are approximately 1 μ M for the conditions and PC1 β -lactamases used (50 mM phosphate, pH 7.0). The analyses utilized data that were collected with remaining nitrocefin concentration significantly higher than the K_M (i.e., nitrocefin concentration > 10 μ M).

Analysis of the kinetic measurement data

Because the binding complexes are not extremely tight, it was necessary to incorporate both association and dissociation processes in the analysis of the experimental kinetic data. Equation 2 is a differential equation for the kinetics including the second order bimolecular association and the first order unimolecular dissociation processes. The rate constants were determined by fitting the time course of the active enzyme concentration to a solution of the following bimolecular binding differential equation,

$$\frac{d[PC-1]_t}{dt} = -k_{on}[PC-1]_t[BLIP]_t + k_{off}[PC-1/BLIP]_t \quad (2)$$

where k_{on} and k_{off} are association and dissociation rate constants, $[PC-1]_t$, $[BLIP]_t$, and $[PC-1/BLIP]_t$ are the concentrations of free unbound PC-1 protein, of free unbound BLIP protein, and of bound PC-1/BLIP complex at time t, respectively. $[PC-1]_t$ was experimentally determined as described above. $[BLIP]_t$ and $[PC-1/BLIP]_t$ were then calculated based on known total concentrations of BLIP and PC-1 proteins. The analytical solution (equation 3) was derived using Maple 12 software (Maplesoft of Waterloo, ON, Canada) as previously described 10. Numerical fitting of the solution to the differential equation (equation 3) yielded the k_{on} and k_{off} values.

$$[PC - I]_t = \frac{-k_{on} \times D - k_{off} + \frac{(e^{(t \times \sqrt{P1})} \times P2 + P2 + e^{(t \times \sqrt{P1})} - 1) \times \sqrt{P1}}{(e^{(t \times \sqrt{P1})} \times P2 - P2 + e^{(t \times \sqrt{P1})} + 1)}}{2 \times k_{on}} \quad (3)$$

where D, P1, and P2 are intermediate variables defined as following:

$$D = [BLIP]_{total} - [PC - I]_{total} \quad (3a)$$

$$P1 = 4 \times k_{on} \times k_{off} \times [PC - I]_{total} + k_{on}^2 \times D^2 + 2 \times k_{on} \times D \times k_{off} + k_{off}^2 \quad (3b)$$

$$P2 = \frac{2[PC - I]_0 k_{on} + k_{on} D + k_{off}}{\sqrt{P1}} \quad (3c)$$

where $[BLIP]_{total}$ and $[PC-1]_{total}$ are total concentrations of BLIP and PC-1 proteins in the experiments. k_{on} , k_{off} , and $[PC-1]_0$ are the association, dissociation rate constants and initial concentration of active PC-1 which are determined by fitting equation 3 to the data. The data processing was carried out using an Excel spreadsheet and the fitting of equation 3 to the data was achieved using the solver add-in of the Excel program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant AI32956 to T.P.

References

1. Strynadka NCJ, Jensen SE, Johns K, Blanchard H, Page M, Matagne A, Frere JM, James MNG. Structural and kinetic characterization of a β -lactamase-inhibitor protein. *Nature*. 1994; 368:657–660. [PubMed: 8145854]
2. Zhang Z, Palzkill T. Dissecting the protein-protein interface between beta-lactamase inhibitory protein and class A beta-lactamases. *J Biol Chem*. 2004; 279:42860–42866. [PubMed: 15284234]
3. Doran JL, Leskiw BK, Aippersbach S, Jensen SE. Isolation and characterization of a β -lactamase-inhibitory protein from *Streptomyces clavuligerus* and cloning and analysis of the corresponding gene. *J Bacteriol*. 1990; 172:4909–4918. [PubMed: 2203736]
4. Thai W, Paradkar AS, Jensen SE. Construction and analysis of beta-lactamase-inhibitory protein (BLIP) non-producer mutants of *Streptomyces clavuligerus*. *Microbiology*. 2001; 147:325–335. [PubMed: 11158349]
5. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. 1995; 39:1211–1233. [PubMed: 7574506]
6. Reichmann D, Cohen M, Abramovich R, Dym O, Lim D, Strynadka NCJ, Schreiber G. Binding hot spots in the TEM1-BLIP interface in light of its modular architecture. *J Mol Biol*. 2007; 365:663–679. [PubMed: 17070843]

7. Reichmann D, Rahat O, Albeck S, Meged R, Dym O, Schreiber G. The modular architecture of protein-protein binding interfaces. 2005; 102:57–62.
8. Reynolds KA, Hanes MS, Thomson JM, Antczak AJ, Berger JM, Bonomo RA, Kirsch JF, Handel TM. Computational redesign of the SHV-1 β -lactamase/ β -lactamase inhibitor protein interface. *J Mol Biol.* 2008; 382:1265–1275. [PubMed: 18775544]
9. Reynolds KA, Thomson JM, Corbett KD, Bethel CR, Berger JM, Kirsch JF, Bonomo RA, Handel TM. Structural and computational characterization of the SHV-1 beta-lactamase-beta-lactamase inhibitor protein interface. *J Biol Chem.* 2006; 281:26745–26753. [PubMed: 16809340]
10. Wang J, Palzkill T, Chow DC. Structural insight into the kinetics and DeltaCp of interactions between TEM-1 beta-lactamase and beta-lactamase inhibitory protein (BLIP). *J Biol Chem.* 2009; 284:595–609. [PubMed: 18840610]
11. Yuan J, Huang W, Chow DC, Palzkill T. Fine mapping of the sequence requirements for binding of beta-lactamase inhibitory protein (BLIP) to TEM-1 beta-lactamase using a genetic screen for BLIP function. *J Mol Biol.* 2009; 389:401–412. [PubMed: 19389404]
12. Zhang Z, Palzkill T. Determinants of binding affinity and specificity for the interaction of TEM-1 and SME-1 β -lactamase with β -lactamase inhibitory protein. *J Biol Chem.* 2003; 278:45706–45712. [PubMed: 12933802]
13. Petrosino J, Rudgers G, Gilbert H, Palzkill T. Contributions of aspartate 49 and phenylalanine 142 residues of a tight binding inhibitory protein of β -lactamases. *J Biol Chem.* 1999; 274:2394–2400. [PubMed: 9891008]
14. Strynadka NCJ, Jensen SE, Alzari PM, James MNG. A potent new mode of β -lactamase inhibition revealed by the 1.7Å X-ray crystallographic structure of the TEM-1-BLIP complex. *Nature Struct Biol.* 1996; 3:290–297. [PubMed: 8605632]
15. Herzberg O, Moul J. Bacterial resistance to beta-lactam antibiotics: Crystal structure of beta-lactamase from *Staphylococcus aureus* PC1 at 2.5 Å resolution. *Science.* 1987; 236:694–701. [PubMed: 3107125]
16. Olsen JE, Christensen H, Aarestrup FM. Diversity and evolution of *blaZ* from *Staphylococcus aureus* and coagulase-negative staphylococci. *J Antimicrob Chemother.* 2006; 57:450–460. [PubMed: 16449305]
17. Deurenberg RH, Vink C, Kalenic S, Freidrich AW, Bruggeman CA, Stobberingh EE. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microb Infect.* 2007; 13:222–235.
18. Arias CA, Murray BE. Antibiotic-resistant bugs in the 21st century- a clinical super-challenge. *N Engl J Med.* 2009; 360:439–443. [PubMed: 19179312]
19. Fuda CCS, Fisher JF, Mobashery S. β -lactam resistance in *Staphylococcus*: the adaptive resistance of a plastic genome. *Cell Mol Life Sci.* 2005; 62:2617–2633. [PubMed: 16143832]
20. Kernodle DS, Stratton CW, McMurray LW, Chipley JR, McGraw PA. Differentiation of beta-lactamase variants of *Staphylococcus aureus* by substrate hydrolysis profiles. *J Infect Dis.* 1989; 159:103–108. [PubMed: 2783329]
21. Rosdahl VT. Localization of the penicillinase gene in naturally occurring *Staphylococcus aureus* strains. *Acta Pathol Microbiol Immunol Scand Sect B.* 1985; 93:383–388. [PubMed: 3879655]
22. Skov RL, Williams TJ, Pallesen L, Rosdahl VT, Espersen F. Beta-lactamase production and genetic location in *Staphylococcus aureus*: introduction of a β -lactamase plasmid in strains of phage group II. *J Hosp Infect.* 1995; 30:111–124. [PubMed: 7673684]
23. Volardi RKR, Kernodle DS. Characterization of a chromosomal gene encoding type B β -lactamase in phage group II isolates of *Staphylococcus aureus*. *Antimicrob Agents and Chemother.* 1998; 42:3163–3168. [PubMed: 9835509]
24. Chan PT. Nucleotide sequence of the *Staphylococcus aureus* PC1 β -lactamase. *Nucleic Acids Res.* 1986; 14:5940. [PubMed: 3488540]
25. Herzberg O. Refined crystal structure of β -lactamase from *S. aureus* at 2 Å resolution. *J Mol Biol.* 1991; 217:701–719. [PubMed: 2005620]
26. Virden R, Qi X. Efficient catalysis of β -lactamase from *Staphylococcus aureus* accompanied by accumulation of an acyl-enzyme. *Biochem J.* 1996; 315:537–541. [PubMed: 8615826]

27. Zawadzke LE, Smith TJ, Herzberg O. An engineered *Staphylococcus aureus* PC1 beta-lactamase that hydrolyses third-generation cephalosporins. *Protein Eng.* 1995; 8:1275–1285. [PubMed: 8869640]
28. Huang W, Zhang Z, Palzkill T. Design of potent β -lactamase inhibitors by phage display of β -lactamase inhibitory protein. *J Biol Chem.* 2000; 275:14984–14988.
29. Chen CCH, Herzberg O. Relocation of the catalytic carboxylate group in class A β -lactamase: the structure and function of the mutant enzyme Glu166-Gln:Asn170-Asp. *Prot Engineer.* 1999; 12:573–579.
30. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol.* 1995; 177:4121–4130. [PubMed: 7608087]
31. Huang W, McKevitt M, Palzkill T. Use of the arabinose P_{bad} promoter for tightly regulated display of proteins on bacteriophage. *Gene.* 2000; 251:187–197. [PubMed: 10876095]
32. Goletz S, Christensen PA, Kristensen P, Blohm D, Tomlinson IM, Winter G, Karsten U. Selection of large diversities of antiidiotypic antibody fragments by phage display. *J Mol Biol.* 2002; 315:1087–1097. [PubMed: 11827478]
33. Kristensen P, Winter G. Proteolytic selection for protein folding using filamentous bacteriophages. *Fold Des.* 1998; 3:321–328. [PubMed: 9806934]
34. Deshayes K, Schaffer ML, Skelton NJ, Nakamura GR, Kadkhodayan S, Sidhu SS. Rapid identification of small binding motifs with high-throughput phage display: Discovery of peptidic antagonists of IGF-1 function. *Chem Biol.* 2002; 9:495–505. [PubMed: 11983338]
35. Schreiber G, Fersht AR. Rapid, electrostatically assisted association of proteins. *Nat Struct Biol.* 1996; 3:427–431. [PubMed: 8612072]
36. Selzer T, Schreiber G. Predicting the rate enhancement of protein complex formation from the electrostatic energy of interaction. *J Mol Biol.* 1999; 287:409–419. [PubMed: 10080902]
37. Carter PJ, Winter G, Wilkinson AJ, Fersht AR. The use of double mutants to detect structural changes in the active site of the Tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell.* 1984; 36:835–840. [PubMed: 6705048]
38. Wells JA. Additivity of mutational effects in proteins. *Biochemistry.* 1990; 29:8509–8517. [PubMed: 2271534]
39. Jones S, Thornton JM. Principles of protein-protein interactions. *Proc Natl Acad Sci USA.* 1996; 93:13–20. [PubMed: 8552589]
40. Bosshard HR, Marti DN, Jelesarov I. Protein stabilization by salt bridges: concepts, experimental approaches and clarification of some misunderstandings. *J Mol Recognit.* 2004; 17:1–16. [PubMed: 14872533]
41. Clackson T, Ultsch MH, Wells JA, de Vos AM. Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity. *J Mol Biol.* 1998; 277:1111–1128. [PubMed: 9571026]
42. Fersht AR. Conformational equilibria in -and -chymotrypsin. The energetics and importance of the salt bridge. *J Mol Biol.* 1972; 64:497–509. [PubMed: 5023185]
43. Hendsch ZS, Tidor B. Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Sci.* 1994; 3:211–226. [PubMed: 8003958]
44. Pons J, Rajpal A, Kirsch JF. Energetic analysis of an antigen/antibody interface: alanine scanning mutagenesis and double mutant cycles on the HyHEL-10/lysozyme interaction. *Protein Sci.* 1999; 8:958–968. [PubMed: 10338006]
45. Wang J, Zhang Z, Palzkill T, Chow DC. Thermodynamic investigation of the role of contact residues of β -lactamase-inhibitory protein for binding to TEM-1 β -lactamase. *J Biol Chem.* 2007; 282:17676–17684. [PubMed: 17430899]
46. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr Biol Crystallogr.* 2004; 60:2126–2132.
47. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. *Nucl Acids Res.* 2003; 31:3497–3500. [PubMed: 12824352]

Figure 1.

A. Illustration of BLIP (green cartoon) bound to TEM-1 β -lactamase (gray spacefill) from the X-ray structure 1JTG 14. The BLIP Asp49 and Phe142 residues are shown in red and are present on two loops that insert into the active site of TEM-1 β -lactamase. **B.** The position of the 23 residues on the BLIP structure that were randomized to create the BLIP phage display library are labeled and shown in purple. BLIP residues that were not randomized are colored yellow. The 23 BLIP residues chosen for randomization are in contact with β -lactamase in the BLIP-TEM-1 β -lactamase structure 14. **C.** An alignment of sequences of several class A β -lactamases. The aligned residues are those that are in contact with BLIP based on the X-

ray structure of the TEM-1 β -lactamase-BLIP complex 14. The alignment was created using the ClustalW2 program 47.

Figure 2.

A. Phage display of the 23 pooled BLIP random libraries. Two rounds of biopanning were performed using immobilized *S. aureus* PC1 β -lactamase as the target. After two rounds of panning, ELISA with immobilized β -lactamase was performed on randomly selected phage clones and those exhibiting binding were subjected to DNA sequencing. **B.** The pTP378 phagemid vector was constructed for display of the BLIP libraries fused to gene III protein on the surface of M13 phage. The BLIP-gIIIp fusion is under the transcriptional control of the arabinose promoter and is only made upon the addition of arabinose.

Figure 3.

Inhibition assays for the interaction between K74G BLIP with *S. aureus* PC1 β -lactamase and its A104E mutant. Inhibition assays were performed by measuring the amount of free β -lactamase at a series of K74G BLIP concentrations. The heavy solid line (triangles) represents inhibition of A104E PC1 β -lactamase by K74G BLIP and the light solid line (circles) represents inhibition of wild type PC1 by K74G BLIP. The data points were fit by nonlinear regression as described in Experimental Procedures. The constants for inhibition of PC1 β -lactamase and A104E PC1 by BLIP and BLIP K74G are listed in Table 2.

Figure 4.

Isothermal titration calorimetric measurements of binding between BLIP and K74G BLIP with PC1 and A104E PC1 β -lactamase at 30°C. The top panels are the raw data and the bottom panels are the integrated heat associated with each experiment. **A.** A104E PC1- β -lactamase (166 μ M) injected into the K74G BLIP mutant (7 μ M). **B.** A104E PC1- β -lactamase (166 μ M) injected into wild type BLIP (7 μ M). **C.** Injection of wild type BLIP (66 μ M) into wild type PC1- β -lactamase (5 μ M). **D.** Injection of K74G BLIP mutant (57 μ M) into wild type PC1- β -lactamase (5 μ M). Both the A104E PC1 β -lactamase/BLIP and the K74G BLIP mutant/PC1 β -lactamase binding isotherms have a sharper transition from the binding region to the saturation region than that of the wild type BLIP/PC1 β -lactamase and of A104E PC1 β -lactamase/K74G BLIP mutant binding isotherms, reflecting the tighter binding of the K74G BLIP mutant/PC1 β -lactamase and of the A104E PC1 β -lactamase/BLIP interactions. All binding events are enthalpically favorable at this temperature (30°C). The binding thermodynamics for these experiments are listed in Table 2.

Figure 5.

Model of PC1 β -lactamase docked with BLIP. The figure shows CPK representations of the specificity determining residues 73 and 74 of BLIP in contact with residue 104 of PC1 β -lactamase. BLIP is represented as a silver cartoon, and PC1 β -lactamase is represented as a red cartoon. Panel **A** shows a model of residue 74 of BLIP as glycine and residue 104 of PC1 β -lactamase as glutamate. Panel **B** depicts lysine 74 of BLIP and alanine 104 of PC1 β -lactamase. Panel **C** illustrates lysine 74 of BLIP and glutamate 104 of PC1 β -lactamase, and panel **D** shows glycine 74 of BLIP and alanine 104 of PC1 β -lactamase. The boxed A104 and K74 listings refer to the residue positions in PC1 β -lactamase and BLIP and the (0, -, +) symbols refer to the charge state of the residue.

Figure 6.

Representative kinetic time courses of association and dissociation of the complexes of PC1 β -lactamase and BLIP proteins. **A.** A typical time course of the intrinsic fluorescence photon counts ($\lambda_{ex} = 277$ nm with 4nm bandwidth and $\lambda_{em} = 330$ nm with 8 nm bandwidth) of the stopped-flow mixing of an equal volume of 5 μ M PC1 A104E and 5 μ M BLIP K74G proteins. This time course is an average of 12 raw traces. The fluorescence signals are represented as percent of intrinsic fluorescence photon count ($\langle I_{eq} \rangle$) of the sample at equilibrium. The red curve is the fitted curve (see Experimental Procedures). **B.** A time course of active enzyme concentration after mixing 20 nM of wild type PC1 and 50 nM of K74G BLIP. At various designated time points after mixing of wild type PC1 and K74G

BLIP, aliquots of the mixture were added with substrate nitrocefin to determine the active enzyme concentration, the solid triangles, as described in Experimental Procedures. The line is the fitted curve as described in Experimental Procedures. **C.** A typical time course of nitrocefin hydrolysis after 400-fold dilution of 10 μ M PC1 A104E/BLIP K74G complex. The black dashed curve is the optical density at 482 nm (with the scale on the right), and the red open diamond symbols are the active enzyme concentrations (with the scale on the left) calculated from the slope of the black curve as described in the Experimental Procedures section. The blue line is the fitted curve. The results of the curve-fitting are tabulated in Table 3.

Figure 7.

A diagram of the double mutant cycle of the changes of binding free energy associated with the mutations of residue 74 of BLIP and of residue 104 of PC1 β -lactamase. **A.** Double mutant cycle for PC1 A104/BLIP K74 wild type to the PC1 A104E/BLIP K74G double mutant. The numerical values next to the boxes represent binding free energy in the units of kcal/mol and the numerical values next to the arrows are the changes of binding free energy ($\Delta\Delta G$). **B.** Calculation of the coupling energy ΔG_I . The coupling energy is obtained from the difference between the sum of the free energy change associated with each single mutant ($\Delta\Delta G_X$, $\Delta\Delta G_Y$) compared to wild type versus the free energy change of the double mutant ($\Delta\Delta G_{XY}$) compared to wild type 37: 38. **C.** Similar calculation of the coupling energy ΔG_I for the double mutation cycle of PC1 A104/BLIP K74 wild type to the PC1 A104E/BLIP K74A double mutant.

Table 1

BLIP mutant sequences obtained after two rounds of biopanning on immobilized *S. aureus* PC1 β -lactamase target protein.

BLIP substitution	Codon usage	Number of occurrences
K74G	GGT	5
K74G	GGA	3
K74G	GGC	3
K74G	GGG	1
E31Q	CAA	1
W150I	ATC	1

Binding thermodynamics associated with the interactions between PC1 β -lactamase or A104E PC1 and BLIP K74G or K74A BLIP variants.

Table 2

	K_d (ITC)	ΔG (ITC at 30 °C)	ΔH (30 °C)	$-T \Delta S$ (30 °C)	K_i (inhibition assay)
PC1 _{wt} /BLIP _{wt}	380 ± 30 nM	-8.9	-5.2 ± 0.2	-3.7	350 ± 100 nM
PC1 _{wt} /BLIP _{K74A}	71 ± 20 nM	-9.9	-7.8 ± 0.3	-2.1	129 ± 30 nM
PC1 _{wt} /BLIP _{K74G}	26 ± 8 nM	-10.5	-9.0 ± 0.2	-1.5	42 ± 20 nM
PC1 _{A104E} /BLIP _{wt}	20 ± 8 nM	-10.7	-13.9 ± 0.6	3.2	24 ± 10 nM
PC1 _{A104E} /BLIP _{K74A}	650 ± 100 nM	-8.6	-12.4 ± 0.5	3.8	750 ± 200 nM
PC1 _{A104E} /BLIP _{K74G}	900 ± 200 nM	-8.4	-16.5 ± 0.2	8.1	500 ± 200 nM

Note: ΔG , ΔH and $-T\Delta S$ are in the unit of kcal/mol

Table 3

Binding kinetic rate constants of interactions between PC1 β -lactamase, BLIP and the A104E PC1 and K74G BLIP variants.

	$k_{on}^{(a)}$	k_{off}	k_{off}/k_{on}
PC1 _{wt} /BLIP _{wt}	$1.0 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$	$1.4 \times 10^{-1} \text{ s}^{-1}$	$1.4 \times 10^{-7} \text{ M}$
PC1 _{wt} /BLIP _{K74G}	$9.8 \pm 2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ $9 \pm 3 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \text{ (b)}$	$4.6 \times 10^{-3} \text{ s}^{-1}$	$4.7 \times 10^{-9} \text{ M}$
PC1 _{A104E} /BLIP _{wt}	$4.3 \pm 0.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ $9.7 \pm 4 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \text{ (b)}$	$1.3 \times 10^{-3} \text{ s}^{-1}$	$3 \times 10^{-9} \text{ M}$
PC1 _{A104E} /BLIP _{K74G}	$2.7 \pm 0.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$	$7.8 \times 10^{-2} \text{ s}^{-1}$	$2.9 \times 10^{-7} \text{ M}$

(a) Association value was determined by following changes in fluorescence in a stopped-flow fluorescence spectrometer.

(b) Association value was determined using an enzyme activity based method.