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Computational Redesign of the SHV-1 β -lactamase/ β -lactamase Inhibitor Protein Interface

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Summary

β -lactamase/ β -lactamase Inhibitor Protein (BLIP) complexes are emerging as a well characterized experimental model system for studying protein-protein interactions. β -lactamases are enzymes that catalyze the hydrolysis of β -lactam antibiotics. BLIP is a 165 amino acid protein that inhibits several class A β -lactamases with a wide range of affinities: pM affinity for K1; nM affinity for TEM-1, SME-1, and BlaI but only μ M affinity for SHV-1 β -lactamase. The large differences in affinity coupled with the availability of extensive mutagenesis data and high resolution crystal structures for the TEM-1/BLIP and SHV-1/BLIP complexes make them attractive systems for the further development of protein engineering and computational design methodologies. We used EGAD, a physics-based computational design program, to redesign BLIP with the goal of increasing affinity for SHV-1. The resulting designed sequences are highly similar to wildtype, with the exception of BLIP residues surrounding β -lactamase position 104. Interestingly, this residue is a known specificity determinant between TEM-1 and SHV-1. Characterization of several of the designs and point mutants revealed that in all cases, the mutations stabilize the interface by 10 to 1000 fold relative to wildtype BLIP. The calculated changes in binding affinity for the mutants were within a mean absolute error of 0.87 kcal/mol from the experimental values, and comparison of calculated and experimental values for a set of 30 SHV-1/BLIP complexes yielded a correlation coefficient of 0.77. Although binding specificity for SHV-1 *versus* TEM-1 was not explicitly considered in the design process, two of the BLIP variants exhibit a small specificity switch. Structures of the two highest affinity complexes, SHV-1/BLIP (E73M) and SHV-1/BLIP (E73M, S130K, S146M), are presented at 1.7 Å resolution. While the predicted structures have much in common with the experimentally determined structures, they do not coincide perfectly; in particular a salt bridge between SHV-1 D104 and BLIP K74 is observed in

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the experimental structures, but not in the predicted design conformations. This discrepancy highlights the difficulty of modeling salt bridge interactions with a protein design algorithm that approximates sidechains as discrete rotamers. Nevertheless, while local structural features of the interface were sometimes miscalculated, EGAD is globally successful in designing complexes with increased affinity.

Keywords

SHV-1 β -lactamase; β -lactamase Inhibitor Protein (BLIP); computational protein design; EGAD; protein-protein interactions

Introduction

SHV-type β -lactamases from *Klebsiella pneumoniae*, hydrolyze penicillins and cephalosporins and are largely responsible for bacterial resistance to these antibiotics. Thus, new SHV-1 inhibitors are desperately needed to combat resistance to β -lactam antibiotics in these gram negative bacteria¹. β -lactamase inhibitor protein (BLIP) competitively inhibits several class A β -lactamases, including SHV-1 and TEM-1. SHV-1 shares 67% sequence identity with TEM-1, and the crystal structures of the unbound β -lactamases overlay with an α -carbon RMSD of 1.4 Å. Yet, the dissociation constant for the SHV-1/BLIP complex is in the micromolar, rather than the nanomolar range as for the TEM-1/BLIP complex². Gaining a quantitative understanding of the interactions underlying BLIP affinity and specificity for the β -lactamases is an important step towards the development of new potent inhibitors. Here, we use computational protein design to study this interface, and to engineer BLIP variants with increased affinity for SHV-1. The TEM-1 β -lactamase/BLIP complex is well studied both experimentally and computationally. These investigations include mutagenesis studies of protein interface modularity^{3; 4}, rational design experiments⁵, a protein-protein docking benchmark⁶, electrostatics calculations to predict association rate constants^{3; 4; 7}, and investigations of protein association in polymer solutions⁸. Notably, several alanine scanning mutagenesis studies were undertaken to determine the specificity determinants for BLIP association with TEM-1, SHV-1, SME-1 and BlaI β -lactamases. Experimental dissociation constants have been determined for more than 120 TEM-1/BLIP mutant complexes, and over 25 SHV-1/BLIP mutant complexes^{2; 3; 4; 5; 9; 10; 11; 12; 13; 14}. High resolution crystal structures of both the TEM-1/BLIP and SHV-1/BLIP complexes are available^{12; 15}. This extensive collection of structural and experimental data is invaluable for informing the present computational design efforts.

In previous work, we conducted mutagenesis and crystallographic studies of the SHV-1/BLIP complex to define the underlying basis for the 1000-fold difference in BLIP affinity for SHV-1 and TEM-1. β -lactamase residue 104 (Ambler residue numbering) is one of eight amino acid differences between SHV-1 and TEM-1 at the β -lactamase /BLIP interface¹⁶. Mutating this residue from aspartate (the residue identity in SHV-1) to glutamate (the residue identity in TEM-1) results in a 1000-fold enhancement in the affinity of BLIP for SHV-1¹². It was hypothesized that the small increase in side chain volume from Asp to Glu allowed improved formation of van der Waals (vdW) contacts and salt bridge interactions

across the interface with BLIP, as seen in the TEM-1/BLIP structure. In particular, a salt bridge between TEM-1 E104 and BLIP K74 is absent in the analogous SHV-1/BLIP structure (Figure 1).

Given that β -lactamase position 104 is now established as a major specificity determinant for the free energy of association of BLIP with SHV-1 *versus* TEM-1, we investigate here if the opposing side of the interface may be redesigned to compensate for the reduced volume of D104 in SHV-1. Can computational methods be used to suggest BLIP mutations in the vicinity of D104 that increase affinity for SHV-1? And further, if BLIP is redesigned to bind with high affinity to SHV-1, what effect will this have on the affinity and specificity of BLIP for TEM-1? The designed BLIP mutants presented here bind SHV-1 with nanomolar affinity, while wild type BLIP exhibits micromolar affinity for SHV-1. Though the designed BLIP variant with the highest affinity for SHV-1 retains a low nanomolar K_{diss} for TEM-1, several of the other designs show a modest specificity reversal, in that they associate with SHV-1 approximately five times more tightly than they do with TEM-1.

Structures of the SHV-1/BLIP (E73M) and SHV-1/BLIP (E73M, S130K, S146M) complexes at 1.7 Å resolution were solved in order to help evaluate the accuracy of the design predictions. Despite a good correlation between the calculated and experimentally determined effects of the design mutations, the crystal structures were not in perfect agreement with the computationally predicted conformations, highlighting specific limitations in our design methodology.

Results

Computational Interface Redesign

The SHV-1/BLIP interface was redesigned with the EGAD software library¹⁷. This method requires a fixed protein backbone, and optimizes the sequence and rotamer conformation of the sidechains according to a physics based energy function parameterized on a large set of protein interface binding affinity data^{18; 19}. A two-state design process was implemented that compares the stabilities of the bound SHV-1/BLIP complex to that of the free species. In this algorithm, the bound and unbound states of the complex are conformationally optimized for each interface mutation, and the energy difference between the states is calculated. The value of this energy difference is maximized throughout the design process. This differs from a typical design run, which only considers the stability of the bound complex, and does not perform side chain conformational optimizations following each mutation (See materials and methods and Figure 2 for details). As the two-state method is computationally expensive, it was necessary to redesign smaller sections of the interface separately to reduce processor time and to improve convergence of the search algorithm. Reichmann et al. showed earlier that the TEM-1/BLIP interface is divisible into energetically independent modules or residue clusters⁴. They demonstrated experimentally that the changes in binding affinity upon mutation ($\Delta G_{diss, mut}$) are highly cooperative for combinations of mutants within a module, and are additive for combinations of mutations in separate modules. The decomposability of the interface suggests that individual clusters can potentially be treated as separate design problems. Here, as a preliminary evaluation of our design software on the β -lactamase/BLIP interface, EGAD was used to redesign the five

interface clusters (C1–C5) described by Reichmann et al.⁴ that are listed in Table 1. A later publication from Reichmann et al. added a sixth interface cluster, but this publication was not available when the design work presented here was initiated³.

For each cluster, the amino acid identities composing the BLIP side of the interface were varied, while the sequence of the β -lactamase remained fixed. Residues in both proteins underwent conformational optimization. Separate designs were conducted for BLIP association with TEM-1 and with SHV-1. The C1 cluster contains only a single BLIP residue, which participates in four hydrogen bonding interactions with SHV-1. This cluster was not redesigned, as this interaction is highly important for interface affinity¹⁰. BLIP position E73 was added to the C2 cluster as it is an important specificity determinant for SHV-1 vs. TEM-1 binding affinity, and is in the immediate vicinity of β -lactamase position 104 (Figure 1). An additional cluster referred to as C2_Plus was created by including BLIP residues S130 and S146 in the C2 cluster. These residues were also selected because of proximity to position 104.

Ten BLIP designs were generated for clusters C2–C5 and C2_Plus, where each cluster was optimized for association with either TEM-1 or SHV-1 (Table 2). In three cases the design recapitulated the WT sequence. For the TEM-1/BLIP designs, the EGAD-proposed BLIP mutations are conservative, and yield little change in the calculated free energy of association with TEM-1. As the wildtype TEM-1/BLIP complex has a $K_d = 1.3$ nM, the interface residues may already be near optimal. Interestingly, redesign of BLIP for association with SHV-1 also yields conservative mutations, with the exception of the C2 cluster. Both the C2 and C2_Plus designs include several mutations predicted to increase affinity for SHV-1 and decrease affinity for TEM-1. These computational results coincide with the experimental observation that the C2 cluster (and in particular β -lactamase residue 104) is a major specificity determinant. Thus, the C2 cluster designs were selected for experimental characterization.

Experimental Determination of Dissociation Constants

The values of the C2 and C2_Plus BLIP/SHV-1 dissociation constants are summarized in Table 3. The C2 design yields an approximately 10-fold increase in affinity compared to wildtype, while the C2_Plus design results in an ~100-fold augmentation. Several point mutants and combinations of design mutations were characterized in order to define the roles of the individual mutations. The F142W, Y143F, and S146M proteins expressed poorly, and therefore were not tested. The S130K mutation stabilizes the SHV-1/BLIP complex by -1.5 kcal/mol relative to wildtype. Interestingly, the E73M point mutant increases complex stability more than either C2 design, resulting in a -3.5 kcal/mol improvement. This implies that one or more of the mutations included in the designs adversely affects complex stability. Given this result, we considered the effects of the other mutations in the E73M context. The E73M/S130K double mutant has a somewhat decreased affinity for SHV-1 compared to E73M alone. The E73M/S146M double mutant also results in little change in K_d from the E73M point mutant, and both the E73M/Y143F and E73M/F142W/Y143F variants have considerably higher K_d values relative to BLIP E73M. Thus,

the E73M point mutation appears to drive the improved affinity of the designed BLIP variants to SHV-1.

TEM-1/BLIP dissociation constants were determined for BLIP mutants that exhibit high SHV-1 affinity. In contrast to the results obtained with SHV-1, all of the characterized variants decreased the affinity of the TEM-1/BLIP complexes. The E73M point mutant destabilizes the TEM-1/BLIP complex the least (0.5 kcal/mol). This is consistent with the computational design of BLIP for TEM-1, which includes the E73L mutation suggesting that a hydrophobic residue at this position is desirable. Although the remaining mutations provide little additional stability to the SHV-1/BLIP complex, they do contribute to complex specificity: the E73M/S130K, E73M/S130K/S146M, and the C2 plus designs associate with SHV-1 ~5-fold more tightly than they do with TEM-1.

Comparison of Experimental and Calculated Changes in Binding Affinity Upon Mutation

The results for the designs are shown together with literature data for a total of 36 observations (Figure 3)^{2, 12}. A correlation coefficient of $R=0.77$ and a mean absolute error of 1.2 kcal/mol was calculated when the six indicated outliers were excluded. The error is increased to 1.9 kcal/mol, and $R=0.52$ when the outliers are included. The outlier points are identical to those identified in our previous work¹², and include the following mutant complexes: SHV-1/BLIP (Y50A), SHV-1/BLIP (Y50A, Y143A), SHV-1/BLIP (E73A, Y50A), SHV-1/BLIP (W112A, Y143A), SHV (D104E)/BLIP, and SHV-1/BLIP (F142A). It was suggested that the destabilization of these complexes is overestimated because many of these mutations involve large changes in side chain volume (Y50A, W112A, F142A) and thus may effect changes in backbone conformation. The EGAD algorithm assumes a fixed backbone: therefore mutations affecting backbone conformation are not accurately modeled.

None of the designed complexes are considered to be outliers. The mean absolute error for the design mutations alone is 0.9 kcal/mol, which is lower than the error for the full data set. The stabilizing effects of the two highest affinity BLIP variants were underestimated. The BLIP E73M mutation was shown experimentally to stabilize the SHV-1/BLIP complex by -3.5 kcal/mol relative to the wildtype, in comparison to a calculated value of -1.4 kcal/mol. Similarly, the experimental and calculated values of $G_{\text{diss, mut}}$ for BLIP (E73M, S130K, S146M) are -3.5 and -1.8 kcal/mol respectively.

Determination of Crystallographic Structures for Mutant Complexes

Structures were determined for the two most stable SHV-1/BLIP complexes: E73M and E73M/S130K/S146M, to 1.75 Å and 1.70 Å resolution respectively. The crystals used for the determination contained the same symmetry, P6₃, and possess nearly identical unit cell dimensions (see Table 4) as the previous wild type structure; though the crystals formed under slightly different conditions. There is little to no change in backbone structure, with an α -carbon RMSD to the wild type of 0.2 Å for both the E73M complex and the triple mutant. The RMSD between the wild type and mutant interface side chains is 0.7 Å for both the E73M and E73M/S130K/S146M complexes. However, unlike the native structure, both mutant complexes include a salt bridge between SHV-1 D104 and BLIP K74, like that seen between TEM-1 E104 and BLIP K74 in the wildtype TEM-1/BLIP complex (Figure 4). The

E73M mutation appears to remove an electrostatic clash with D104, allowing the salt bridge to form. In the TEM-1/BLIP complex, the longer E104 side chain is able to avoid this clash, and form the E104/K74 salt bridge in the presence of E73. This observation is consistent with the proposal of Zhang and Palzkill, offered to explain the alanine scanning mutagenesis results for E73². The E73A mutation results in very little change in K_d for the TEM-1/BLIP complex, while E73A causes a -1.97 kcal/mol stabilization in the SHV-1/BLIP complex. The E73M mutation is more stabilizing than E73A, perhaps because E73M is a more conservative substitution with respect to side chain volume. Additionally, a lower dielectric environment due to the methionine side chain may enhance the affinity of the salt bridge.

Comparison of the predicted design conformations with the experimental structures provides an explanation for the underestimated stabilizing effects of E73M in the design calculations. The computationally predicted conformations fail to include the D104/K74 salt bridge, and thus neglect this energetic contribution to the interface. In the predicted structure of the triple mutant, D104 is seen to point toward S130K (Figure 4b), while in the predicted structure of E73M, D104 retains the conformation seen in the wildtype protein (Figure 4a). Additionally, the conformation of Y143 is incorrectly placed in the triple mutant – the designed structure predicts that Y143 will be pushed out of the interface by the S146M mutation, while the experimental structure shows that it remains in the native conformation. The conformations of the remaining side chains are fairly well-described by the calculated structures. Comparison of the predicted and experimental side chain conformations within a 6 Å radius of the D104/K74 salt bridge yields RMSDs of 0.7 Å and 1.2 Å for E73M and E73M/S130K/S146M respectively.

Discussion

The computational design protocol resulted in two designs (C2 and C2_Plus) with increased SHV-1 affinity. The calculated changes in free energy of association for the design mutations reproduce the experimental values within an average error <1 kcal/mol. The design algorithm was successful in terms of these global measures, but closer scrutiny by mutagenesis and structural characterization revealed a significant flaw in the predicted structures of the designs. A single mutation, E73M, is responsible for most of the increase in stability. The crystal structures show that this mutation facilitates the formation of a salt bridge between D104 (SHV-1) and K74 (BLIP), a feature that escaped the calculation procedure. Why was this salt bridge overlooked? And how can the calculation be improved to capture this feature more accurately? The potential sources of error and strategies for increasing the accuracy of the design methodology are discussed below.

It is important to mention that the predicted values of $G_{\text{diss, mut}}$ for the designs and design point mutants span a range of 1.3 kcal/mol, close to the observed error in our calculations. Thus, only an approximate rank ordering of designs and point mutations is expected. Several contributing factors including inaccuracies in the energy function, the fixed backbone assumption, the discrete rotamer representation of sidechains, and a reduced search space that includes only a subset of the interface residues might be responsible. The striking factor however is the failure to identify the effect of the E73M mutation on the formation of the D104/K74 salt bridge. Two reasons may contribute to the incorrectly predicted

conformation of D104/K74: 1) inaccuracies in the energy function, causing the stabilizing effects of the salt bridge to be underestimated relative to other interactions; 2) lack of proper rotamer conformations to model salt bridge formation. Importantly, when the crystallographic sidechain conformations of D104 and K74 are included as part of the rotamer library, and the SHV-1/BLIP E73M backbone is used as a template, the salt bridge is correctly predicted by EGAD rotamer optimization. Conversely, when the crystallographic conformations are not included, or the backbone template is taken from the wildtype SHV-1/BLIP structure (rather than from the mutant complex) the salt bridge is not identified. The closest pair of sidechains from the rotamer library exhibits a sidechain RMSD of 0.5 Å to the experimental conformation. The relative frequency of these rotamers within the PDB was calculated using Coot; the K74 rotamer has a frequency of 3%, and the D104 rotamer a frequency of 21%²⁰. Neither of these rotamers is considered rare. While the crystallographic sidechain conformations are not conformationally distinct enough from the rotameric conformations to qualify as new rotamer entries within the library, building the D104/K74 rotamer pair onto the wildtype SHV-1/BLIP complex backbone (used as the template for the designs) further increases the RMSD to 0.6 Å (Figure 5a). This displacement increases the distance between the K74 amide nitrogen and D104 oxygens, disfavoring the salt bridge. To further assess how slight differences in backbone conformation impact the outcome of the design predictions, the crystallographic rotamers from the E73M structure were built onto the wildtype SHV-1/BLIP backbone. This yields an RMSD of 0.3 Å when compared with the crystallographic conformation of the salt bridge (Figure 5b). A deviation of 0.2 Å in the backbone conformation between the wildtype and E73M structures brings the amide nitrogen of K74 and od2 oxygen of D104 closer together by 0.6 Å. As such, a van der Waals (vdW) clash is introduced, and the salt bridge is not correctly identified during rotamer optimization. In a separate test, the D104/K74 sidechain pair on the wildtype template was optimized to minimize the RMSD between the wildtype conformation and the E73M crystal structure conformation (Figure 5b). This minimization was conducted using the ICM software package, and allowed both the $\alpha/\alpha/\beta$ bond distance and planar $\alpha/\alpha/\beta$ bond angle to be varied, as well as the torsional χ angles²¹. The variation in bond distance and planar bond angle was included to compensate for the slight differences in backbone conformation. This optimization resulted in a sidechain pair on the wildtype template with an RMSD of 0.1 Å to the E73M crystal structure. Rotamer optimization of the interface with this rotamer pair correctly identified the salt bridging interaction.

Neither the rotamer library conformations nor the protein backbone used in the original design predictions deviate by more than 0.6 Å from the experimentally determined conformation. However, these structural changes were sufficient to preclude discovery of the correct designed interface conformation. The difficulties in calculating accurate vdW energies with a fixed backbone and rotamers are well documented; our findings suggest that these problems may become particularly acute for salt bridges or hydrogen bonds²². In these situations a delicate balance must be achieved - the residues must come close enough to form a stabilizing interaction, but not so close that they create a vdW clash. The EGAD forcefield parameters dictate that ideal hydrogen bonding distances are shorter the sum of the atomic vdW radii. Indeed, in the E73M crystal structure, the amide nitrogen of K74 and

carboxyl oxygen of D104 are positioned at a distance of 2.8 Å, while the sum of their respective vdW radii is 3.3 Å. During design calculations, this sidechain conformation generates a vdW clash that must be compensated for by an energy term favoring electrostatic or hydrogen bonding interactions. If rotamers in the library do not capture the exact geometry necessary to favor electrostatic interaction without generating an overwhelmingly destabilizing vdW clash, the salt bridge will be eliminated as a false negative.

Increased sidechain flexibility would improve the prediction of salt bridge interactions by providing conformations consistent with salt bridge formation and reducing the number of salt bridges overlooked as “false negatives”. One possible means of achieving greater sidechain flexibility is through the use of an expanded rotamer library. This method greatly increases computation time, and has been shown to provide little advantage over using a softened vdW potential²⁰. Another possibility is the use of continuous optimization techniques, which allow for full side chain flexibility rather than discrete rotamers. These methods require an analytically differentiable energy function, which typically precludes the inclusion of a solvation term. Unlike fully flexible side chains, discrete rotamers conveniently allow for the precalculation of pairwise energy tables, which can improve calculation speed. Further, some search algorithms require a pairwise-decomposable energy function, and thus also the use of discrete rotamers (for example DEE). For these reasons, several groups have suggested hybrid approaches that couple iterations of continuous local minimization (allowing fully flexible sidechains) with global optimization algorithms (that use discrete rotamers). The local minimization process allows incorporation of continuous sidechain flexibility and “off-rotamer” sidechain conformations, but is limited to exploring conformational space near the closest local minimum. Global optimization ensures that high energy barriers separating low energy conformations may be crossed, and allows inclusion of non-analytically differentiable energy terms. A two-tier Monte Carlo simulation was used to recently used to redesign a protein/peptide interface²³. This simulation introduced a random change in sequence identity or rotamer conformation. Next, local minimization considering continuous sidechain conformations was performed. The energy obtained by local minimization was then used in the Monte Carlo simulation to accept or reject the move in sequence or conformation space. Similarly, the biased probability Monte Carlo (BPMP) algorithm developed in the Abagyan lab performs a round of local minimization for each Monte Carlo step²¹. In this algorithm, a random change in sequence identity or rotamer conformation is introduced. Local minimization is then performed using only the analytically differentiable terms of the energy function. The energy of the new conformation is then calculated using the full energy function, and used to accept or reject the Monte Carlo move. This was used successfully in the refinement of sidechain conformations for protein-protein docking calculations. In this paper, Monte Carlo optimization was used to select new interface sequences and DEE was used to optimize interface sidechain conformation. Future design calculations may also include a brief round of local minimization, such as conjugate-gradient, following DEE. This would allow evaluation of “off-rotamer” conformations, and possibly improve the EGAD design results.

Materials and Methods

Protein Expression and Purification

SHV-1, TEM-1 and BLIP were expressed and purified as described in^{12; 24}.

Experimental Determination of K_d Values

β -lactamases were assayed with the substrate nitrocefin (ncf, Becton Dickinson, Cockeysville, MD). BLIP and β -lactamase were mixed in sodium phosphate buffered saline (PBS) containing 100 μ g/ml bovine serum albumin. β -lactamase concentrations were 3–10 nM, with the lower concentrations used for studies of the higher affinity inhibitors. Reactions were initiated with ncf at the K_m value for the enzyme (15 μ M for SHV-1 and 350 μ M for TEM-1). Final reaction volumes were 1 ml, and all measurements were performed in triplicate. In order to minimize variation between protein preparations, all measurements were performed with a single pooled sample of either SHV-1 or TEM-1. Hydrolysis rates were monitored at $\lambda = 486$ nm using the extinction coefficient, $\epsilon = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$ for the hydrolyzed form of ncf (ϵ taken from Calbiochem product literature).

BLIP inhibition curves were plotted with Origin® 7.5 SR software and were fit to the quadratic equation:

$$E_{free} = [E_0] - \frac{[E_0] + [I_0] + K_d^* - \sqrt{([E_0] + [I_0] + K_d^*)^2 - (4[E_0][I_0])}}{2}$$

where E_{free} is the remaining free enzyme concentration calculated from activity, $[E_0]$ is initial enzyme concentration, $[I_0]$ is the BLIP concentration and K_d^* is the apparent equilibrium constant¹¹. K_d^* was corrected for the presence of substrate using the equation:

$$K_d = \frac{K_d^*}{(1 + [S]/K_m)}$$

Crystallography

SHV-1 was mixed 1:1 with BLIP E73M, or BLIP (E73M, S130K, S146M) in 20 mM BisTris pH 7.25, 50 mM NaCl and concentrated. The concentrated protein was dialyzed overnight against 20 mM BisTris pH 7.25, 50 mM NaCl. The final concentration of protein used for setting trays was either 4.1 mg/ml SHV-1/BLIP E73M or 6.3 mg/ml SHV-1/BLIP (E73M, S130K, S146M). Crystals were grown at 19 °C in hanging drop format by mixing 1 μ l protein with 1 μ l well solution or 2 μ l protein with 2 μ l well solution over a 1ml reservoir of well solution. Both the SHV-1/BLIP E73M and SHV-1/BLIP (E73M, S130K, S146M) complexes formed hexagonal crystals in well solutions of 37.5% or 42.5% ammonium sulfate, 100 mM Tris pH 8.4. For reference, our previous crystals of the wild type complex were obtained in 60% ammonium sulfate, 50 mM cacodylate pH 6.5¹². For harvesting, a cryoprotectant solution containing well solution plus 30% xylitol was added directly to the drop, and the crystals were immediately looped and flash-frozen in liquid nitrogen.

All datasets were collected at Beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory²⁵. Data were indexed and scaled using HKL2000²⁶. Subsequently, molecular replacement was performed using PHASER with the wild type SHV-1:BLIP structure (PDB entry 2G2U) as search model²⁷. Iterations of manual rebuilding in Coot, and refinement with PHENIX generated the final structural models^{20; 28}. Additional details of the data collection and refinement are provided in Table 4. All molecular figures were created with PyMOL²⁹.

Computational Interface Design

All design calculations were performed using the EGAD library for computational protein design¹⁷. The protein backbone was held fixed to the crystallographic conformation of the SHV-1/BLIP complex (PDB ID: 2G2U). Amino acid identity was sampled for sidechains composing a single interface cluster (further described in the results), and a second shell of residues within 4 Å of the designed positions was varied rotamerically. The Dunbrack backbone independent rotamer library was used for all calculations, with added 180° flips of χ_2 for asparagine, glutamine, and histidine³⁰. For sidechains with fewer than three χ angles, χ_1 was also spread by one sigma in each direction. All amino acid identities with the exception of G, P and C, were allowed at the designed positions. The energy function used is that described in^{18; 19}, and consists of a linearized vdW term, coulombic electrostatics, and torsional potentials, all using atomic parameters from OPLS-AA^{31; 32}. The Generalized Born model and a solvent accessible surface area dependent hydrophobic burial term were included to incorporate solvation effects.

Previously, EGADlib free energy calculations were shown earlier to reproduce experimental changes in free energy upon interface mutation within an error of 1.6–2.2 kcal/mol for the TEM-1/BLIP and SHV-1/BLIP interfaces. These calculations explicitly considered the energy of the bound complex relative to a reference state of unbound monomers. As this improved the agreement with experimental values, the unbound state energy was incorporated as a reference state in the designs presented here. The design process minimized G_{diss} , rather than the energy of the bound complex alone:

$$\Delta G_{\text{diss}} = G_{\text{bound}} - G_{\text{free}}$$

The free monomers were modeled by separating the crystallographic structure of the complex by 50 Å. A two state minimization protocol was employed, consisting of an outer search algorithm that conducted optimization in sequence space, and an inner minimization loop that optimizes in structure space. The Monte Carlo method was used as the outer minimizer. Minimization was initiated by random selection of an amino acid position and introduction of a mutation. The inner minimization loop (here Dead End Elimination) then performed rotamer optimization of both the bound and unbound states of the new mutant complex. The difference in energy between the bound complex and unbound monomers was returned to the outer Monte Carlo loop. The Monte Carlo minimization subsequently accepted or rejected the mutational move in accordance with the Metropolis criterion, and randomly selected another position for mutation. As the Monte Carlo method is a heuristic search algorithm, ten independent minimization runs were performed, and all ten designs

underwent a heuristic quench local minimization step. In this process, a single amino acid position was randomly selected and mutated to all possible residue identities to identify the lowest energy residue. This was done in random order across the designed residues until all positions had been visited at least once with no change in residue identity. Following this minimization, it was found that the ten design runs converged on 1 to 3 individual solutions. The modular structure of the EGAD library greatly facilitated nesting and combination of the search algorithms in the two state minimizer implementation, and will later allow for experimentation with other search algorithm combinations such as genetic algorithm with DEE.

There are two ways to introduce mutations with overall stabilizing effects on the interface in two state design procedures: 1) introduce a mutation that stabilizes the bound state or 2) add one that destabilizes the unbound monomers. Although both approaches do result in a greater free energy difference between the bound and unbound states, the latter approach often generates design solutions that are calculated to improved binding affinity, but fail to do so. For example, large vdW clashes are easily introduced into the unbound state as a consequence of the fixed backbone approximation. While this gives rise to the desired effect on G_{diss} , in reality these clashes would be relaxed, or cause unfolding of the unbound monomers. To mitigate this problem, the vdW term used to describe the unbound monomers is softened slightly relative to the term describing the bound complex. Specifically, the linearization point of the repulsive potential is shifted to a larger inter-atomic distance. The equation describing the softened vdW potential is:

If $r_{ij} \leq \sigma_{ij}\beta$:

$$E_{ij}^{\text{vdW}} = \frac{4\omega_{ij}}{\beta^6} \left(\frac{6}{\sigma\beta} \left(1 - \frac{2}{\beta^6} \right) r_{ij} + \frac{13}{\beta^6} - 7 \right)$$

If $\sigma_{ij}\beta < r_{ij} < \sigma_{ij}$:

$$E_{ij}^{\text{vdW}} = 4\omega_{ij} \frac{\sigma_{ij}^6}{r_{ij}^6} \left(\frac{\sigma_{ij}^6}{r_{ij}^6} - 1 \right)$$

If $r_{ij} > \sigma_{ij}$:

$$E_{ij}^{\text{vdW}} = 4\mu\omega_{ij} \frac{\sigma_{ij}^6}{r_{ij}^6} \left(\frac{\sigma_{ij}^6}{r_{ij}^6} - 1 \right)$$

where :

$$\beta = 1 - \zeta(2^{1/6} - 1)$$

For the bound state of the complex, $\zeta = 1.5$, while for the free monomers $\zeta = 1.0$. The value for the bound state was taken from¹⁸, while the value for the free monomers was selected by visually inspecting designed complexes at different ζ thresholds.

Calculation of $G_{\text{diss, mut}}$ Values

The equation used for comparison with experimental values is:

$$\Delta\Delta G_{\text{diss, mut}} = \eta(\Delta G_{\text{diss, mut}} - \Delta G_{\text{diss, WT}})$$

The scaling factor η is necessary because EGAD consistently returns much larger values of $G_{\text{diss, mut}}$ than those observed experimentally. For all $G_{\text{diss, mut}}$ calculations, $\eta = 0.20$. This value was empirically determined by fitting to a large set of experimental interface mutation data¹⁸. The change in affinity upon mutation was calculated with an energy function identical to that used for the present designs. The crystallographic complexes of TEM-1/BLIP and SHV-1/BLIP (PDB ID: 1JTG, 2G2U) were used as the starting structures for bound state energy calculation, and the unbound states were modeled by separating the complex structures by 50 Å. As in the design process, for each mutant complex a subset of amino acid sidechains was rotamer optimized for the bound and unbound states by DEE (thus guaranteeing a global minimum), and a free energy calculated for the minimum energy state. As the free energy of association calculations are less computationally demanding than a full design run, more side chains were included in the rotamer optimization. This set includes all cluster residues (instead of the side chains for only a single cluster at a time), and all positions with one atom within 4 Å of any of the cluster residues. All amino acid positions with the identities G, P and C were held fixed in conformation. This change in search space yields some small differences between the energies for the designs calculated here and those calculated in the design runs. However, in all cases the differences in calculated energy are well within the error we observe between the calculated and experimental values.

Protein Data Bank Accession Codes

The Protein Data Bank accession codes for the SHV-1/BLIP E73M complex and SHV-1/BLIP E73M/S130K/S146M complex are 3C4P and 3C40, respectively.

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Abbreviations

BLIP	β -lactamase Inhibitor Protein
vdW	van der Waals
ncf	nitrocefin
DEE	Dead-End Elimination

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Figure 1.

Comparison of β -lactamase residue 104 and BLIP Lys 74 in TEM-1 and SHV-1. β -lactamase is shown in a grey cartoon representation, and BLIP in blue. The global context of the salt bridge formed between these two residues is shown in panel (a). A close-up view of the interaction, as boxed in panel (a), is provided in panel (b). In the wildtype TEM-1/BLIP complex, E104 and K74 form a salt bridge (yellow sidechains) that is not observed between SHV-1 D104 and K74 (cyan sidechains).

Figure 2.
Schematic of the two state optimization procedure used to redesign the SHV-1/BLIP interface.

Calculated vs. Experimental $\Delta\Delta G$

Figure 3.

Correlation between experimental and calculated values of $G_{\text{diss, mut}}$. Each point on the plot represents a mutant SHV/BLIP complex for which the change in binding affinity relative to wild type has been experimentally measured. Several of the complexes are point mutants arising from alanine scanning mutagenesis studies, while others include up to five mutations. The designed complexes and point mutants were both computationally predicted and experimentally measured to increase the binding affinity of the SHV/BLIP complex, and fall in the lower left quadrant of the graph (red squares). For several complexes, the

destabilizing effects of mutation were overestimated by our model; these are denoted as outliers and are shown in open black diamonds.

Figure 4.

Comparison of the computationally predicted and experimentally-determined structures of the designed SHV-1/BLIP complexes. In each panel, the SHV backbone is shown in grey ribbons, and the BLIP backbone in blue ribbons. Panel (a) details the interface sidechain interactions for the predicted (cyan sticks) and experimental (yellow sticks) conformations of SHV-1/BLIP E73M. Similarly, panel (b) shows the predicted (orange sticks) and experimental (green sticks) conformations of the E73M/S130K/S146M SHV/BLIP complex. In each case, the calculated structure fails to predict the D104/K74 salt bridge seen in both

crystal structures. BLIP loop residues 44–51 and 138–144 are removed for clarity, with the exception of Y143, as it is observed in a different conformation between the predicted and experimental structures.

Figure 5.

Comparison of crystallographic and rotamer library sidechain conformations. In both panels, the crystallographic sidechain conformations for the D104/K74 salt bridge in the E73M mutant structure are shown in magenta, and the interatomic distances labeled in red. In panel (a), the rotamer library pair with the lowest RMSD to the crystallographic conformation is shown built on the E73M backbone template (cyan), and on the wild type complex structure (green), which was used as the template for the designs. The interatomic distances for the wild type complex are labeled in blue. A combination of variations in sidechain and

backbone conformation result in an increased distance between the salt bridge pair in the wild type structure. In panel (b), the crystallographic sidechain conformation is shown built onto the wildtype backbone (orange). For this sidechain pair, the interatomic distance between the amide lysine and one of the carboxyl oxygens is shortened (blue labels), introducing a vdW clash. The sidechain pair with α/β bond distance, α/β bond angle, and torsional χ angles optimized for RMSD to the crystallographic conformation is displayed in yellow.

Table 1

Modular interface clusters for the TEM-1/BLIP complex, from Ref 4.

Cluster	TEM-1 positions	BLIP positions
C1	S130, K234, S235, R243	D49
C2	N170, E104, Y105, A237	A47, G48, K74, G141, F142, Y143
C3	Q99, N100, L102, V103, P167, E168	W112, H148, W150, R160, W162
C4	P107, M129, V216	F36, H41, Y50, Y53
C5	E110	S71, S113

Table 2

Calculated substitutions for the association of BLIP with TEM-1 and SHV-1.

<i>Interface design results for BLIP binding to TEM-1:</i>				
Cluster:	Designed Positions: (BLIP Only)	Design Result:	Calculated $G_{\text{diss, mut}}$ for TEM-1*	Calculated $G_{\text{diss, mut}}$ for SHV-1*
C2	A47/E73/K74/F142/Y143	E73L	−0.53	−0.96
C2_Plus	A47/E73/K74/S130/F142/Y143/S146	No convergence.	--	--
C3	W112/H148/W150/R160/W162	WT sequence	0	0
C4	F36/H41/Y50/Y53	WT sequence	0	0
C5	S71, S113	S113M	−0.43	−0.59
<i>Interface design results for BLIP binding to SHV-1:</i>				
C2	A47/E73/K74/F142/Y143	E73M/F142W/Y143F	7.61	−1.90
C2_Plus	A47/E73/K74/S130/F142/Y143/S146	E73M/S130K/F142W/Y143F/S146M	10.54	−2.71
C3	W112/H148/W150/R160/W162	WT sequence.	0	0
C4	F36/H41/Y50/Y53	Y53F	4.3	−0.17
C5	S71, S113	S113N	−0.14	−0.05

* The $G_{\text{diss, mut}}$ values shown were calculated using EGAD with the larger side chain search space employed in the binding affinity change predictions, rather than the smaller search space used during the design runs (see materials and methods for details).

Table 3

Experimentally determined dissociation constants for the BLIP C2 design and C2 design variants in complex with SHV-1 and TEM-1.

BLIP Mutation	K _{diss} (nM) SHV-1	G _{bind} SHV-1 (kcal/mol)	K _{diss} (nM) TEM-1	G _{bind} TEM-1 (kcal/mol)
WT	1720 ± 60	0	1.3 ± 0.1	0
C2 (E73M/F142W/Y143F)	265 ± 14	−1.11 ± 0.04	n.d.	n.d.
C2_Plus (E73M/S130K/F142W/Y143F/S146M)	12.2 ± 0.7	−2.93 ± 0.04	79 ± 2.3	2.43 ± 0.02
C2_P1 (E73M/S130K/F142W/Y143F)	57.2 ± 1	−2.01 ± 0.02	n.d.	n.d.
C2_NoW (E73M/Y143F)	117 ± 4	−1.59 ± 0.03	n.d.	n.d.
E73M	4.4 ± 0.6	−3.53 ± 0.08	3.2 ± 0.1	0.53 ± 0.02
S130K	144 ± 4.0	−1.47 ± 0.03	12.7 ± 1.6	1.35 ± 0.07
E73M/S130K	15.0 ± 0.5	−2.81 ± 0.02	50 ± 3	2.2 ± 0.4
E73M/S130K/S146M	4.6 ± 0.2	−3.51 ± 0.03	27 ± 2	1.8 ± 0.4

Table 4

Crystallographic data collection, refinement and stereochemistry

	SHV-1:BLIP E73M	SHV-1: BLIP E73M S130K S146M
Data Collection:		
Resolution (Å)	1.75 – 48	1.70 – 48
Wavelength (Å)	1.1159	1.1159
Space Group	P 63	P 63
Unit Cell Dimensions (a,b,c) Å	127.48, 127.48, 73.41	127.23, 127.23, 73.42
Unit Cell Angles (α,β,γ)°	90.0, 90.0, 120.0	90.0, 90.0, 120.0
I/s (last shell)	30.9 (3.1)	20.1 (2.7)
R _{sym} (last shell) %	0.078 (0.626)	0.098 (0.442)
Completeness (last shell) %	100.0 (100.0)	98.0 (84.9)
No. of reflections	1530836	1152849
unique	68483	74627
Refinement:		
Resolution (Å)	1.75 – 36	1.70 – 36
No. of reflections	68431	73095
working	64967	69413
free (% total)	3464 (5.1)	3682 (5.0)
R _{work} (last shell) (%)	18.7 (24.7)	17.1 (21.7)
R _{free} (last shell) (%)	21.0 (28.7)	20.0 (23.0)
Structure and Stereochemistry:		
No. of atoms	3757	3779
protein	3257	3263
water	490	506
RMSD bond lengths (Å)	0.005	0.004
RMSD bond angles (°)	0.963	0.981

$$R_{sym} = \frac{\sum_j \sum_i |I_j - \langle I \rangle|}{\sum_j I_j}$$
 , where I_j is the intensity measurement for reflection j and $\langle I \rangle$ is the mean intensity for multiple recorded reflections.

$$R_{work, free} = \frac{\sum ||F_{obs}| - |F_{calc}||}{|F_{obs}|}$$
 , where the working and free R -factors are calculated using the working and free reflection sets, respectively. The free reflections were held aside throughout refinement.