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# Protein–protein interaction: A genetic selection for compensating mutations at the barnase–barstar interface

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**ABSTRACT** **Barnase and barstar are trivial names of the extracellular RNase and its intracellular inhibitor produced by *Bacillus amyloliquefaciens*. Inhibition involves the formation of a very tight one-to-one complex of the two proteins. With the crystallographic solution of the structure of the barnase–barstar complex and the development of methods for measuring the free energy of binding, the pair can be used to study protein–protein recognition in detail. In this report, we describe the isolation of suppressor mutations in barstar that compensate for the loss in interaction energy caused by a mutation in barnase. Our suppressor search is based on *in vivo* selection for barstar variants that are able to protect host cells against the RNase activity of those barnase mutants not properly inhibited by wild-type barstar. This approach utilizes a plasmid system in which barnase expression is tightly controlled to keep the mutant barnase gene silent. When expression of barnase is turned on, failure to form a complex between the mutant barnase and barstar has a lethal effect on host cells unless overcome by substitution of the wild-type barstar by a functional suppressor derivative. A set of barstar suppressors has been identified for barnase mutants with substitutions in two amino acid positions (residues 102 and 59), which are critically involved in both RNase activity and barstar binding. The mutations selected as suppressors could not have been predicted on the basis of the known protein structures. The single barstar mutation with the highest information content for inhibition of barnase (H102K) has the substitution Y30W. The reduction in binding caused by the R59E mutation in barnase can be partly reversed by changing Glu-76 of barstar, which forms a salt bridge with the Arg-59 in the wild-type complex, to arginine, thus completing an interchange of the two charges.**

Much of biological existence is determined by unique interactions at the molecular level. A vast array of molecular recognitions are specific protein–protein interactions between enzymes and their substrates, enzymes and their inhibitors, the assembly of identical or different subunits into larger structures, etc. What is the nature of the interactions involved in protein–protein recognition? Which forces stabilize protein complexes? How plastic are the interfaces? One approach to this problem is to determine which changes in one partner will compensate for weakened binding caused by mutation in the other.

We have developed a system to do just this, using *in vivo* selection to find such complementing mutations. The gene for barnase, the extracellular RNase of *Bacillus amyloliquefaciens*, is lethal when expressed in *Escherichia coli* without concurrent expression of its intracellular polypeptide inhibitor barstar. The genes for both proteins have been cloned in *E. coli* plasmids and both can be produced in quantity as plasmid products (1). As both proteins are small and can be reversibly unfolded *in vitro*, they are particularly well suited to studies of protein folding (2–6) and, together, of protein–protein interaction (7–10). The results of a mutagenic survey revealed that among the mutations in barnase that drastically reduce RNase activity are several that also substantially increase the dissociation constant of the barnase–barstar complex (9, 10). The recent crystallographic structure of this complex (7, 8) confirmed identification of barnase residues His-102 and Arg-59 as being among those directly involved in the interaction.

Due to the toxic nature of barnase, coexpression of barstar is necessary to suppress the lethal effect of expressed active barnase. The wild-type gene for barnase was, for this reason, originally cloned with the gene for barstar on the same plasmid (1). A typical use of this system involves site-directed mutagenesis of the barnase or barstar gene; isolation of mutant protein; and assay for activity, stability, or binding properties. Since this approach requires isolation of significant quantities of mutant proteins, we can expect to have difficulty with mutations that greatly reduce stability and yield of the protein, or, in the case of barnase mutants, that being active but not inhibited by barstar are lethal. Our attempts to produce the H102K mutation in barnase in our barnase production plasmid (pMT416) produced a normal number of clones, all of which, however, grew very slowly and were too unstable to maintain. This was our first clue that we could really identify mutants that were active but poorly inhibited by barstar and inspired development of the suppressor strategy reported here.

## MATERIAL AND METHODS

**Bacterial Strains and Plasmids.** *E. coli* strain D1210 (11) was used for cloning, propagation, and other manipulation of the plasmids. λ lysogenic strain D1210HP (12) was used for heat-induced inversion of the barnase–barstar cassette *in vivo*. *E. coli* HB101 was used for production of barstar mutants. The tightly controlled expression plasmid pMI43a with the reversible barnase–barstar cassette is derived from pMI41a (13) by deletion of t1 transcription terminator from the *attP* site, corresponding to nucleotides 27522–27570 in the conventional map of λ (14). pMI43b is the form of pMI43a with a reversed barnase–barstar cassette. Plasmid pMJ2, used for production of barnase(H102K), has the Y30W suppressor mutation in barstar and was prepared by subcloning the *Aat* II/*Hin*dIII fragment from pMI305 (H102K mutation in barnase and Y30W in barstar in pMI43b) into pMT416 (1) in place of the wild-type genes. Suppressor barstar proteins were produced in the background of both pMT316 (1) and pMT643. pMT643, which produces barstar A, differs from pMT316 only by mutation of the two cysteine residues of barstar to alanine (C40A/C82A). Functionally, barstar A is nearly the equal of the wild-type barstar (9).

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Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

**Inversion of Barnase–Barstar Cassette *in Vivo*.** Int-mediated inversion of the barnase–barstar cassette in plasmid pMI43a was carried out in lysogenic strain D1210HP essentially as described by Podhajska *et al.* (15). Briefly, the plasmid carrying strain was grown at 30°C in LB medium supplemented by ampicillin (100 μg/ml) and induced by heating to 42°C for 15 min. Appropriately diluted culture was spread on LB plates with ampicillin or tested for lethal induction level of barnase expression on plates supplemented by increasing levels of isopropyl β-D-thiogalactopyranoside (IPTG; 4–24 μg/ml). Inversion was verified by restriction analysis of plasmids.

**Mutagenesis Procedures.** Oligonucleotides for site-specific and codon-saturated (random) mutagenesis were prepared on an Applied Biosystems model 380B synthesizer. Site-specific mutagenesis was carried out by the method of recombinant circle PCR described by Jones and Howard (16). This method was adapted for random mutagenesis of one or two codon positions in barstar by using an unbiased NN(G/C) (N is 25% of all four nucleotides, G/C means 50% of G and C) randomization in mutagenized codon positions to generate all of the possible amino acids.

**Suppressor Selection.** In single codon randomization experiments, annealed products were directly transformed into *E. coli* D1210 by electroporation (17), and suppressors were selected on LB plates with IPTG (4 μg/ml). This concentration of IPTG is the minimal level that results in barnase induction lethal for strain D1210 with barnase(H102K) and wild-type barstar genes on the pMI43 plasmid after inversion. The same level of IPTG is the minimal level that is lethal for barnase(H102K) without barstar, suggesting only very weak binding and inhibition, if any, of barnase(H102K) by wild-type barstar. The presence of active barnase(H102K) was confirmed in surviving clones by deletion of the barstar (mutant) gene from their plasmid constructs, followed by transformation and selection under minimal lethal levels of barnase(H102K) induction (IPTG; 4 μg/ml). When two codons were randomly mutagenized, annealed products were digested by *Bam*HI and *Xba* I and the restriction fragment (carrying the barstar mutant library) was recloned into the backbone of the same plasmid, which, however, had not undergone PCR amplification to avoid the possibility of unwanted mutations in the barnase gene. All transformants were pooled and the mixed plasmid population was isolated and retransformed into *E. coli* D1210. Transformants were selected for suppressors on plates with different levels of IPTG (4–16 μg/ml). Mutations in the barstar gene from surviving clones were identified by sequencing double-stranded DNA with Sequenase, version 2.0 (United States Biochemical).

**Other Methods.** Barnase and barstar preparation and determination of dissociation constants ($K_d$) of barnase–barstar complexes have been described (9).

## RESULTS AND DISCUSSION

**Strategy for Searching for Barnase Mutant Suppressors.** The rationale of this strategy is outlined in Fig. 1. The promoterless barnase gene is inserted with the complete barstar gene in the expression plasmid pMI43a in the antisense orientation to prevent a basal level of toxic barnase expression. When heat-induced, this vector will invert the barnase–barstar cassette and barnase will be transcribed by the tandem P*tac-lac* promoters (13). Inversion is mediated by interaction between *attP* and *attB* sites derived from λ phage (15). Since the barnase gene is in the antisense orientation prior to inversion, synthesis of toxic gene product is limited at the transcriptional level by production of barnase-specific antisense RNA. This is particularly important when an active barnase variant (wild type or mutant) is poorly inhibited by barstar or its mutant. Barnase expression in this system is regulated at two levels. First, a brief derepression of the int operon provided by λ lysogenic strain

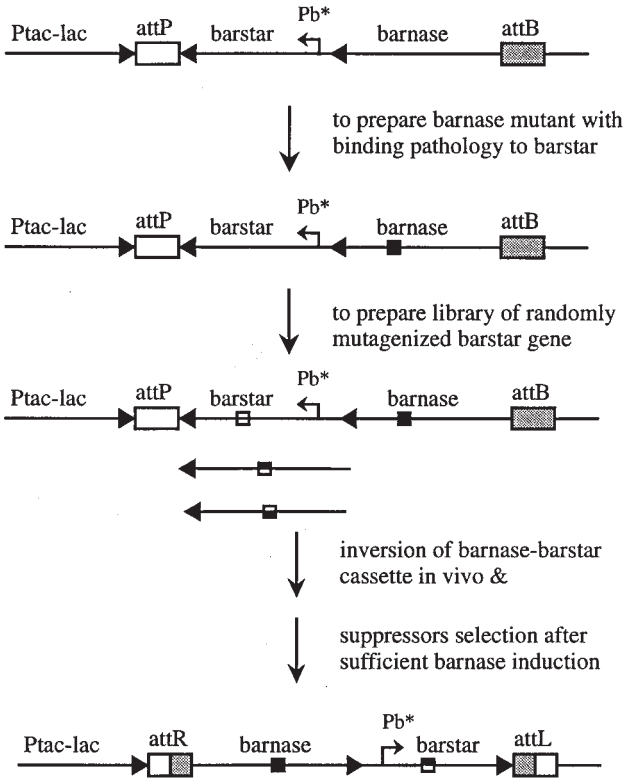


FIG. 1. General strategy for suppressor searching. Barstar suppressors are selected under conditions of barnase expression, where the original barnase (mutant)–barstar pair is lethal. The lethal level of IPTG induction correlates with the binding energy between barnase and barstar variants. If a particular barnase mutation does not cause host cell pathology after inversion of barnase–barstar cassette, we can prepare a mutagenized barstar library in inverted plasmid and select after sufficient IPTG induction. *attP* and *attB* sites are derived from the phage λ attachment site (15). Sites *attR* and *attL* are products of recombination between *attP* and *attB*. Pb* is the barstar natural promoter.

D1210HP results in expression of the int gene of phage λ (15). The int product inverts the barnase–barstar cassette cloned between *attP* and *attB* sites, resulting in expression of barnase from tandem promoters, P*tac*-P*lac*. The second level of regulation is provided by induction of P*tac*-P*lac* by gradually increasing levels of IPTG. Since the barstar gene is on its own promoter, it is constitutively expressed, and reversal of the barnase–barstar cassette (and even induction by IPTG up to 24 μg/ml) will not have a toxic effect on the host unless inhibition is compromised by a mutation in one or the other of the two proteins. If such a mutation interferes with inhibition, the particular plasmid construct may function as a "host killer" after sufficient induction of barnase expression. In this case, we can produce random mutations of other amino acids in the recognition surface of either protein (in our present work in barstar) and select for survivors under conditions of barnase expression where the original mutation is lethal.

**Suppressor Mutations for Barnase(H102K).** It has been shown (7–10) that a significant barnase contribution to the interface area comes from residues His-102 and Arg-59. The catalytic residue, His-102, forms three hydrogen bonds to barstar and its entire side chain fits into a surface barstar pocket (ref. 7; see also Fig. 2). Barnase mutants H102D, H102Q, H102G, H102L, and H102A have no detectable RNase activity (9, 13, 18, 19), and their binding to barstar is greatly reduced. However, a more conservative substitution, H102K, in the active-site prepared in our standard high-copy expression vectors pMT416 (1) and pMT702 (9) produced

slow-growth, plasmid rearrangement-prone transformants. These clones do not produce either RNase activity on RNA plates or inactive barnase in amounts detectable by barnase antiserum on nitrocellulose filters. It was suggested (9), and recently demonstrated *in vivo* (13), that this mutation produces a barnase with residual RNase activity that is not properly inhibited by barstar and thereby limits growth. In the two-stage barnase expression system pMI43a-b (Fig. 1), we are able to keep the barnase(H102K) gene in host cells not only under the most tightly controlled conditions (in antisense orientation prior to inversion) but also after inversion without induction by IPTG. This is presumably due to the lower copy number of all derivatives of pMI43a in comparison with derivatives of pMT416 (or pMT702), which are based on the high copy number vector pUC19. However, after induction with low levels of IPTG (4 $\mu$g/ml), expressed barnase(H102K) is lethal for *E. coli* strain D1210, while transformants with wild-type barnase easily survive due to better protection by barstar. This lethal level of barnase (H102K) was the minimal IPTG induction level for selection of barstar suppressors.

Each of the six residues of barstar that contact His-102 in the native complex (Fig. 2)—Tyr-29, Tyr-30, Gly-31, Asn-33, Ala-36, and Asp-39—were randomly mutagenized. Two barstar mutants, Y29P and Y30W, provided better protection against the RNase activity of barnase(H102K), indicating the importance of the two barstar tyrosine positions (residues 29 and 30) for recognition of barnase(H102K). The double barstar mutant (Y29P/Y30W) prepared by site-directed mutagenesis was also functional in our *in vivo* assay. We next randomly mutagenized both barstar tyrosine residues (29 and 30) simultaneously. From this experiment, the following sets of functional barstar suppressors were obtained: Y29D/Y30W, Y29A/Y30W, Y29N/Y30W, and Y29R/Y30W. One of these experiments also provided the double mutant Y29I/Y30G. Although this barstar mutant can rescue cells from barnase(H102K) under conditions where the construct carrying the barnase(H102K) with the wild type barstar is lethal, its inhibition capacity seems to be much lower than other double mutants. Barstar (Y29D/Y30W) is our best suppressor overall, allowing survival with the highest level of barnase(H102K) induction by IPTG. The list of functional suppressors (Fig. 3) strongly indicates the high significance of tryptophan residue in position 30 in barstar for mutual interaction with barnase(H102K). Since the phenolic group of Tyr-30 in the wild-type complex is completely buried inside the barstar structure and contacts barnase only through its peptide backbone (Fig. 2), we can expect that its replacement by the larger tryptophan is accompanied by rearrangements in barstar structure.

**Reverse Charge Suppressor for Barnase(R59E).** The barnase mutation R59E, in pMT416, produces a phenotype similar to that of H102K, with small, unstable colonies (9). Arg-59 is situated on the edge of the recognition site, is not totally buried in the complex, and forms one salt bridge with

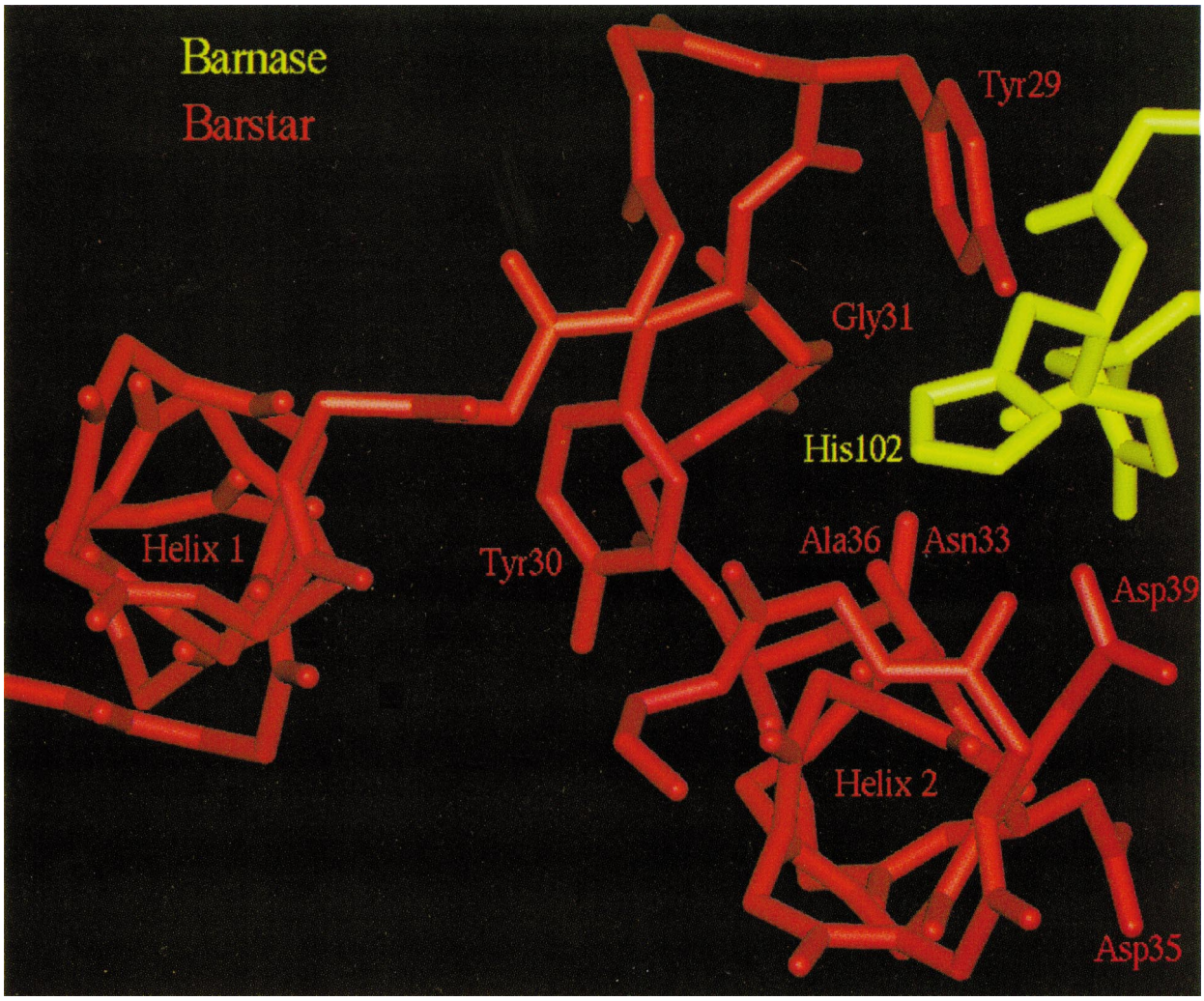


FIG. 2. Active site His-102 of barnase and its barstar environment. Side chain of His-102 (barnase) fits into surface barstar pocket defined by residues Tyr-29, Tyr-30, Gly-31, Asn-33, Ala-36, and Asp-39 (7). Each of these barstar residue positions was randomly mutagenized in order to find suppressor mutations for barnase(H102K).

| aa # | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| wild type | A | L | P | G | Y | Y | G | E | N | L | D | A | L | W | D | C |
| Y29P | - | - | - | - | P | - | - | - | - | - | - | - | - | - | - | - |
| Y30W | - | - | - | - | - | W | - | - | - | - | - | - | - | - | - | - |
| Y29P,Y30W | - | - | - | - | P | W | - | - | - | - | - | - | - | - | - | - |
| Y29A,Y30W | - | - | - | - | A | W | - | - | - | - | - | - | - | - | - | - |
| Y29D,Y30W | - | - | - | - | D | W | - | - | - | - | - | - | - | - | - | - |
| Y29N,Y30W | - | - | - | - | N | W | - | - | - | - | - | - | - | - | - | - |
| Y29R,Y30W | - | - | - | - | R | W | - | - | - | - | - | - | - | - | - | - |
| Y29I,Y30G | - | - | - | - | I | G | - | - | - | - | - | - | - | - | - | - |

FIG. 3. Amino acid residues 25–40 of wild-type barstar and barstar suppressor mutations for barnase(H102K). Shaded amino acids in wild-type barstar are those in contact with His-102 of barnase in the wild-type complex. Dashes indicate amino acid identity with wild-type residues.

Glu-76 of barstar and hydrogen bonds to its Asp-35 (7, 8). Therefore, instead of using selection from a random library, we followed the logic of the situation and found that reversal of the charge of Glu-76 of barstar by a directed mutation (E76R) restored viability under conditions where barnase(R59E) was otherwise lethal. Interchanging the charges between these two interacting positions, then, can restore inhibition and barstar(E76R) protects host cells against barnase(R59E) much better than does wild-type barstar. *E. coli* D1210 cells with barnase(R59E) and the barstar suppressor mutation E76R can survive on LB plates with IPTG (8 $\mu$g/ml) (lethal level of induction for barnase with R59E and wild-type barstar) are able to produce more barnase antigen, and secreted RNase activity is easily detectable on RNA plates (data not shown).

***In Vitro* Study of Barnase–Barstar Binding.** That the lethal effect of barnase mutants such as barnase(H102K) and the compensating effect of our suppressor mutations in barstar relates to relative avidity of the various barnase–barstar pairs for each other seems clear from the *in vivo* results. To remove any doubt, however, it is necessary to isolate the proteins and demonstrate (*i*) that barnase(H102K) actually has RNase activity and (*ii*) that it is bound by, and is inhibited by, a suppressor barstar more strongly than by the wild type. It was only after our discovery of barstar suppressors of the barnase(H102K) pathology that we were able to produce and purify usable quantities of the mutant enzyme. Construction of plasmid pMJ2, carrying barnase(H102K) and barstar(Y30W) allowed production of barnase(H102K) with yields approaching that of the wild type. Subsequent purification was then essentially the same as for the wild type.

It was not possible to demonstrate hydrolytic activity of barnase(H102K) toward our polyethenoadenosine fluorogenic substrate. By measuring the production of acid-soluble material, we could, however, measure activity against RNA. Using the assay procedure of Rushizky *et al.* (20), but raising the temperature to 42°C and extending the incubation time to 1 hr, we found a specific activity of barnase(H102K) of ≈0.1% that of wild type. This activity was completely inhibited by a 2-fold excess of purified barstar A(Y29D/Y30W) but not by even larger amounts of wild-type barstar. Barstar(Y29D/Y30W), without the two cysteine-to-alanine mutations, was not used in this test as its crude preparation was contaminated with enough *E. coli* RNases to mask any inhibition.

The Y29D/Y30W barstars, with and without the C40A/C82A mutations, both bound wild-type barnase weakly enough that their $K_d$ values and concentrations in crude extracts could be determined directly from activity titration curves (9). The $K_d$ values so determined were $1 \times 10^{-10}$ for barstar A(Y29D/Y30W) and $8 \times 10^{-11}$ for barstar(Y29D/Y30W). As noted above, barnase(H102K) does not cleave our assay substrate. If, then, we add some of this protein to a solution of active barnase partly inhibited by a barstar, the measured rate of hydrolysis of the substrate will be increased if barnase(H102K) can compete with the active barnase for the barstar. By combining the mass action relations for the three protein components, we can then determine the $K_d$ for the barstar–inactive barnase pair

$$K_d[b^*,b_i] = \frac{b_{i0}(b_{a0} - b_a)K_d[b^*,b_a]}{(b_a b_0^* - (b_a + K[b^*,b_a])(b_{a0} - b_a))},$$

where $b_a$ is the free active barnase as measured by its activity; $b_{a0}$, $b_{i0}$, and $b_0^*$ are the total concentrations of active barnase, inactive barnase, and barstar, respectively; and $K_d[b^*,b_a]$ is the $K_d$ for the barstar–active barnase complex. For these experiments, it was not necessary, or even desirable, that the active barnase be the wild type, since a mutant barnase with activity but reduced avidity for barstar would allow us to detect a lower level of competition by the inactive barnase(H102K). We therefore used the active mutant barnase(H18R/R59A), which has about a third the activity of the wild type but binds to either barstar or barstar A with a dissociation constant greater by >3 orders of magnitude. The results are summarized in Table 1. As can be seen, the double-mutation Y29D/Y30W reduces the binding of the barstars to wild-type barnase by 2 or 3 orders of magnitude but increases their binding to barnase(H102K) by 4 orders of magnitude or more. With a 200-fold excess of barnase(H102K) over barnase(H18R/R59A) in competition for barstar A we could just barely measure its effect on activity. In the same competition for wild-type barstar, we could set only an upper bound to the effect.

These measurements, combined with detection of RNase activity for barnase(H102K) and its inhibition by the suppressor barstar, leave no doubt that the interpretation of our *in vivo* results in terms of barnase–barstar binding are correct.

## CONCLUSION

Protein–protein interactions play an important role in many biological processes. Several approaches, including structural

Table 1. $K_d$ values for different barnase–barstar combinations

| | Barstar | | | |
|---|---|---|---|---|
| Barnase | Wild type | C40A/C82A | Y29D/Y30W | C40A/C82A and Y29D/Y30W |
| Wild type | $6 \times 10^{-14}$* | $2 \times 10^{-13}$* | $8 \times 10^{-11}$ | $1 \times 10^{-10}$ |
| H18R/R59A | $1.5 \times 10^{-10}$* | $1.7 \times 10^{-10}$* | $5 \times 10^{-9}$ | $1 \times 10^{-9}$ |
| H102K | $>5 \times 10^{-5}$ | $2 \times 10^{-5}$ | $2 \times 10^{-9}$ | $3 \times 10^{-9}$ |

*From Hartley (9).

studies, have been used recently to explore recognition between the bacterial RNase barnase and its polypeptide inhibitor barstar (7–10). Methods of site-specific mutagenesis have allowed us (9) and others (10) to measure the effect of substitutions on the dissociation constant. Genetic approaches to this problem can take the form of using single (or multiple) codon randomization to select functional pairs of interacting proteins. This can provide information about the extent to which particular amino acids in a structure are replaceable. At some residue position, the precise chemical characteristics of a side chain are essential for recognition and no other amino acids will substitute. Conversely, if the chemical identity of the side chain is unimportant, many different substitutions will be permitted. This sort of structural and functional study has been done for other single proteins—e.g., λ repressor (21) or β-lactamase (22). In this report, we initiated studies of the interacting proteins barnase and barstar from the opposite direction. Starting from a malfunctioning pair of proteins, in which recognition was disturbed by a mutation in barnase, we selected suppressor mutations in barstar able to compensate the primary mutation and to improve mutual communication. The property of the barnase–barstar system (lethality and its suppressibility), combined with our system for functional selection of compatible partners, can serve as a powerful tool with which to manipulate and explore a protein–protein interface. The system might also serve as a model for use in other toxin-inhibitor recognition studies.

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