Directed Mutagenesis and Barnase–Barstar Recognition

Robert W. Hartley

Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received October 19, 1992; Revised Manuscript Received March 25, 1993

ABSTRACT: Directed mutagenesis has been applied to the cloned genes of barnase and barstar, the extracellular ribonuclease of *Bacillus amyloliquefaciens* and its intracellular inhibitor, to locate residues involved in the mutual recognition of these two proteins. Arg59 and His102 of barnase and Asp35 and Asp39 of barstar have been so identified. With both Cys40 and Cys82 mutated to alanines, barstar is still produced in high yield and is functional both *in vitro* and *in vivo*. Methods devised for determining relative and absolute dissociation coefficients for various combinations of mutant and wild-type proteins have allowed us to determine a dissociation coefficient for the complex of wild-type barnase and barstar of about 10^{-13} M, with off and on rate constants of 10^{-5} s^{-1} and 10^{8} M^{-1} s^{-1}, respectively.

Barnase is the major extracellular ribonuclease produced by *Bacillus amyloliquefaciens* (Hartley, 1989). Barstar is a specific intracellular inhibitor of barnase, produced by the same organism. Inhibition involves the formation of a stable noncovalent one-to-one complex of the two proteins (Hartley & Smeaton, 1973). The three-dimensional structure of barnase is known, both from X-ray crystallography (Mauguen et al., 1982; Baudet & Janin, 1991) and from NMR studies in solution (Bycroft et al., 1991). The structure of the complex of barnase and a mutant barstar has recently been solved by X-ray crystallography (Y. Mauguen, personal communication). Barnase belongs to a family of ribonucleases of both prokaryotic and eukaryotic origin (Hartley, 1980; Hill et al., 1983). Three residues (in barnase; Glu73, Arg87, and His102) are conserved in the whole family and are among those involved in catalysis.

As both proteins have been cloned and expressed in *Escherichia coli* (Hartley, 1988), site-directed mutagenesis can be used to probe various aspects of their structures, activities, and interactions. This report presents an effort to survey the surfaces of both proteins to determine what portions are involved in the interaction. Since the surface residues of barstar were not known, reversal of charge mutations (e.g., Lys to Glu and vice versa) was carried out on most of its charged amino acids. Since expression of barnase is lethal to *E. coli* in the absence of barstar expression, we would expect to have difficulty in producing mutants which were still active but uninhibited by barstar. In the course of this work, procedures were worked out for measuring off rates and relative and absolute dissociation coefficients for some combinations of native and mutant proteins.

Barnase is a simple linear peptide of 110 amino acids which folds into its active conformation reversibly, without the complications of disulfide bond formation or the need for divalent cations or other nonpeptide components. As such it makes an admirable subject for protein folding studies [e.g., see Prevost et al. (1991), Pace et al. (1992), and Serrano et al. (1992)]. Barstar has only 89 residues, but 2 of these, at positions 40 and 82, are cysteines which may form a disulfide bond even in its intracellular milieu. This complication can be avoided by converting both cysteines to alanine. The resulting barstar can be obtained in high yield and is functional both *in vitro* and *in vivo*, although its complex with barnase is less stable than that of the wild-type. It is the crystal structure of this mutant that has been solved in complex with barnase.

As it seems that barnase is becoming a paradigm for the general problem of protein folding [I quote Prevost et al. (1991)], the barnase–barstar pair is well suited for the same role in the study and design of protein–protein noncovalent interactions and quaternary structure. In addition to investigations on the detailed fit of the interacting surfaces, studies of the sort reported here can model the effects of longer range forces, such as overall charge differences, on both association and dissociation rates. Barstar, for example, still inhibits barnase after conversion to a basic protein by the replacement of as many as three glutamic acids (in several combinations) by lysine (unpublished results).

MATERIALS AND METHODS

Barnase and Barstar Assay. Barnase activity was measured at 25 °C in 0.2 M ammonium acetate/1.0 mM EDTA, pH 8, as the initial rate of fluorescence increase on hydrolysis of polyethenoadenosine phosphate (Fitzgerald and Hartley, unpublished results), using a Perkin-Elmer-Hitachi MPF-3L fluorescence spectrophotometer with incident and emission wavelengths set at 320 and 410 nm, respectively. A standard activity curve (linear up to at least 50 ng/mL) based on purified wild-type barnase, with a molar absorptivity at 280 nm of 2.6×10^{4} M^{-1} cm^{-1}, was used to express all activities as equivalent weights of wild-type barnase. The activity of barstar, which binds and inhibits barnase on a one-to-one basis, was expressed as the weight of barnase inhibited.

Barnase and Barstar Preparation. Barnase and barstar were prepared from cultures of *E. coli* (JM107 or SURE for barnase, HB101 for barstar) carrying the expression plasmids for wild-type or mutant protein. Purification of barnase and its mutants was a modification of that reported for its production from *B. amyloliquefaciens* (Hartley & Robertson, 1972), involving extraction from the culture medium onto SP-Sephacryl-M, elution with 2 M ammonium acetate, pH 8, and buffer-exchange on a Sephadex G25 column to 0.02 M of the same salt at pH 5, followed by ion-exchange gradient chromatography on SP-Sephacryl-M. The ammonium acetate, pH 8, gradient contained 1 mM EDTA. Yields were generally about 20 mg/L for the wild-type enzyme and 100 mg/L for the inactive His102 mutants. For all except some low-yield mutants, the products were essentially pure by SDS–PAGE.

Purification of barstar and its mutants involved the use of an affinity column bearing covalently bound barnase. This was prepared using cyanogen bromide-activated Sepharose 6B (LKB—Pharmacia) according to the manufacturer's directions, with 10 g of the dry material added to 23 mL of ice-cold 0.1 M sodium borate, pH 8.5, containing 50 mg of wild-type barnase. After the material was stirred gently by tumbling overnight in the cold and the addition of 1 mL of 1.0 M ammonium acetate, it was allowed to settle. The A_{280} of the supernatant was negligible, indicating that virtually all of the protein had been bound. Packed in a 0.9-cm by 50-cm column and washed with 0.1 M ammonium acetate/1.0 mM EDTA, pH 8, the column's capacity for barstar was subsequently found to correspond to approximately 40 mg of barnase. After each use, the column was washed with 0.1 M ammonium acetate/1.0 mM EDTA, 1.0 M ammonium acetate/1.0 mM EDTA, and 1.0 mM EDTA, all at pH 8, and then again with the first buffer. It has been stored at room temperature for over 1 year and used repeatedly, without appreciable loss of capacity.

Except where noted, the following procedure was carried out at room temperature. Harvested cells of *E. coli* carrying the barstar production plasmid pMT316 or a mutant thereof were first extracted with approximately 10 times the packed cell volume of acetone and then 3 times with similar volumes of 0.2 M NaCl/1.0 M acetic acid. The final pellet was taken up in a like volume of 0.2 M ammonium acetate/1.0 mM EDTA, pH 8. The suspension was adjusted to pH 8–8.5, allowed to stand for 10 min, and centrifuged. The supernate was held at 4 °C overnight, centrifuged to remove cold-insoluble materials, and then put through an 0.2-μm membrane filter. After assay, the sample, or a fraction of it sufficient to inhibit up to 30 mg of barnase, was applied to the barnase column. The column was washed sequentially with several column volumes each of 0.1 M ammonium acetate/ 1.0 mM EDTA, pH 8, 1.0 M ammonium acetate/1.0 mM EDTA, pH 8, and 1.0 mM EDTA, pH 8, and then again with the first buffer. The barstar was then removed by elution with a 1% solution of sodium dodecyl sulfate (SDS) in the first buffer. Fractions containing the protein peak were pooled and lyophilized. The dry material (mostly SDS) was taken up in a minimum volume of water and dialyzed exhaustively (at least 3 days with several changes of the 1-L dialysate per day) versus 1.0 mM EDTA, pH 8. SDS was not detectable (less than 0.1 mM, or less than 0.05 mol of SDS per mole of barstar) in the dialysand by the method of Reynolds and Tanford (1970).

A rough measure of the yields of barnase and barstar as antigens, particularly useful for inactive mutants, was made by staining either membrane-grown colonies or Western blots with appropriate antisera. The staining and electrophoretic procedures have been reported (Paddon & Hartley, 1987).

Mutagenesis. Site-directed mutagenesis was carried out essentially by the procedure of Jones and Howard (1990), the necessary oligonucleotides being prepared on an Applied Biosytems 380B DNA synthesizer. *E. coli* transformations were carried out by electroporation (Speyer, 1990). This was done with an apparatus which provided for the discharge of a 10-μF capacitor charged to 250 V through the 10-μL sample held between steel plates separated by ca. 180 μm (14 kV/cm). The samples consisted of mid-log-phase cells washed several times in 20% glycerol plus the transforming DNA. Electrodes and sample were at 4 °C during the operation.

In the early part of this work, barnase mutants were produced in the background of pMT416, described previously

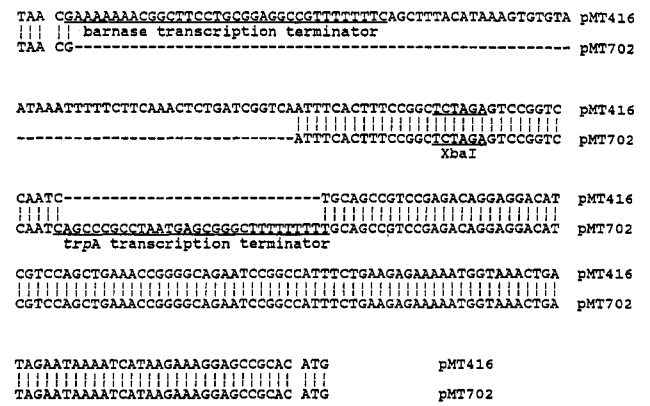

FIGURE 1: Difference between the barnase expression fragments of pMT416 (Hartley, 1988) and pMT702, used for many of the barnase mutants referred to in this report. pMT702 and its derivatives also carry the adventitious mutation H18R.

(Hartley, 1989), or pMT413, which had the same expression fragment carried between the *Eco*R1 and *Hin*dIII sites of pUC19. As the barnase transcription terminator just 3′ to the structural gene contains an inverted repeat that interferes with PCR mutagenesis near the N-terminus of the protein, later work used a derivative, pMT702, in which this was deleted and an *E. coli trp*A transcription terminator inserted further downstream (Figure 1). The background for barstar mutants was pMT316, described previously (Hartley, 1989). Embarrassingly late in the work it was discovered that pMT702 also carried the adventitious barnase mutation H18R. As His18 is a surface residue far removed from the active site, we should not expect its conservative mutation to affect any of the findings reported here. Indeed, I have found no effect on specific activity or, as noted later, on binding to barstar. Dideoxy sequencing of the genes for all the proteins used in the binding studies found no mutations other than those expected, including, where noted, the barnase H18R mutation derived from pMT702.

Barnase–Barstar Reaction: Kinetic and Equilibrium Measurements. Titration of wild-type barnase with wild-type barstar yields a straight line with a very sharp cutoff at zero barnase activity when the barstar concentration equals that of barnase. As the protein concentrations in these titrations are on the order of 10^{-8} M, curvature of the end point would be detectable if the dissociation constant were as high as about 10^{-11} M. For certain combinations of active mutant barnases and mutant or wild-type barstar, the dissociation constants are indeed higher than this and can be measured by fitting the titration curves with an equation based simply on the law of mass action:

$$K_d = [b][b^*]/[bb^*] \quad (1)$$

where K_d is the dissociation constant and [b], [b*], and [bb*] are the concentrations of barnase, barstar, and their complex, respectively. From the conservation of barnase and barstar molecules, we also have

$$[bb^*] = [b]_0 - [b] = [b^*]_0 - [b^*] \quad (2)$$

where $[b]_0$ and $[b^*]_0$ are the total concentrations of barnase and barstar added. Combining eq 1 and 2, we get the simple quadratic equation:

$$[b]^2 + (K_d + [b^*]_0 - [b]_0)[b] - K_d[b]_0 = 0 \quad (3)$$

The experimental data were fit by eye to a family of computer-generated plots of [b] *vs* $[b^*]_0$ to determine K_d.

Mutations of the active-site His102 to any of several other amino acids completely eliminates ribonuclease activity and reduces, but does not eliminate, the molecule's affinity for barstar (Hartley & Fitzgerald, 1989). When such a mutant barnase and an active barnase, mutant or otherwise, are mixed sequentially with barstar, the time course of subsequently measured ribonuclease activity depends on their order of addition. In the experiments reported here, each mixture contained 50 ng/mL active barnase, a slight excess of barstar, and a greater amount of an inactive barnase mutant. Mixtures were maintained at 25 °C, with 2-mL samples transferred to cuvettes in the spectrofluorometer, at the same temperature, just before addition of substrate.

Determination of relative dissociation constants from such experiments carried to equilibrium required much higher concentrations of the inactive barnase mutant and times up to 24 h. By switching the order of addition of the two barnases, equilibrium could be approached from both directions. Enough of the inactive mutant barnase was used to bring the equilibrium value of the free active barnase to greater than 10% of its total. Controls containing the active and inactive barnases but no barstar showed no loss of activity in the equilibration times used. From the measured activity ([b]) at equilibrium and the total concentrations of the three proteins ($[b]_0$ for the active barnase, $[b^-]_0$ for the inactive barnase, and $[b^*]_0$ for barstar) and using the law of mass action, $K_d = [b][b^*]/[bb^*]$, and (with the total barstar concentration equal to that of the active barnase and the free barstar concentration negligible) the conservation relations $[b] = [b^-b^*]$ and $[b]_0 = [bb^*] + [b^-b^*]$, we can get the ratio of the dissociation constants:

$$R_K = K_{active}/K_{inactive} = [b]^2/([b^-]_0 - [b])([b]_0 - [b]) \quad (4)$$

While theoretically more satisfying, the results of these experiments were less precise than the kinetic measurements, especially for the more tightly bound pairs which required many hours for equilibration.

Thermal Unfolding. Thermal unfolding of barnase and its mutants was done in 0.2 M ammonium acetate/1.0 mM EDTA, pH 8, and observed as the decline in tryptophan fluorescence (Hartley, 1975). Barstar is relatively resistant to thermal unfolding, with a T_m in the vicinity of 80 °C in aqueous buffers at pH 8. It was necessary, therefore, to carry out the barstar melting experiments in 3 M urea, 0.1 M ammonium acetate, and 1.0 mM EDTA, pH 8, where the T_m of wild-type barstar is 61 °C. The reversible unfolding reaction was followed, as with barnase, as a decrease in fluorescence due to exposure of Trp residues to solvent (excitation, 279 nm; emission, 310 nm). Extraction of the fraction unfolded versus temperature curves was considerably less precise than for barnase due to the relatively steep drop off of the barstar fluorescence in 3 M urea outside the melting range, but the results were adequate for present purposes.

RESULTS

Barnase Mutants. Table I lists the mutations of residues on the surface of barnase which neither greatly reduce activity or yield nor interfere with inhibition by barstar. As can be seen in this table and, graphically, in Figure 2, nonconservative substitutions can be made over much of the surface of barnase without eliminating activity or fatally interfering with its inhibition by barstar. Clones carrying the barnase surface mutations D54K, D93K, K98D, and D101K (all of which also carried the H18R mutation) produced little or no barnase activity. Of these, only D54K and D101K produced appreciable barnase antigen, and their yields were far below the wild-type.

Table I: Mutations of Barnase Surface Residues Which neither Greatly Reduce Yield or Activity nor Interfere with Inhibition by Barstar

amino acid substitutions	coding sequence	
	wild-type	mutant
V3D,I4D	GTTATC	GATGAT
I4D,N5I	ATCAAC	GATATC
D8K[a]	GAC	AAG
D12I	GAT	ATT
H18R	CAT	CGT
K19E[a]	AAG	GAG
D22S	GAT	TCT
E29K[a]	GAA	AAG
G34V,V36G	GGCTGGGTG	GTCTGGGGG
K39E	AAA	GAA
D44K[a]	GAC	AAG
K49E	AAA	GAA
S57E	TCA	GGA
E60K,K62E[a]	GAAGGCAAA	AAGGGCGAA
K66E	AAA	GAA
S67D	AGC	GAT
R69E[a]	CGA	GAA
N77I[a]	AAC	ATC
T79D[a]	ACA	GAC
T79V[a]	ACA	GTT
S80E	TCA	GAA
F82A[a]	TTC	GCC
S85A[a]	TCA	GCC
D86K[a]	GAC	AAA
Q104K[a]	CAG	AAG

[a] Mutant barnases also carry the mutation H18R derived from the plasmid pMT702 as described in the text.

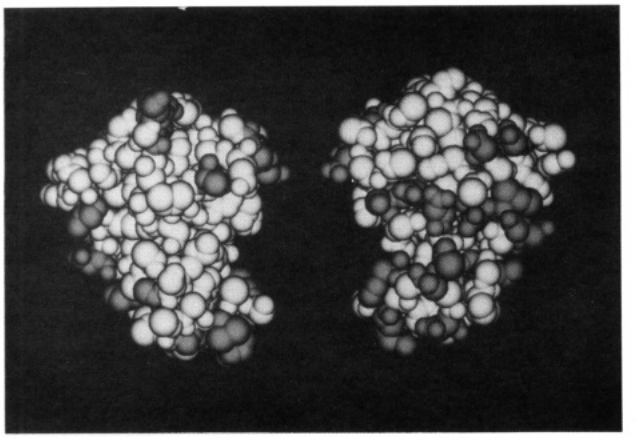

FIGURE 2: Two views 180° apart ("front" and "back") of barnase. Shaded darker are those residues for which nonconservative substitution can be made without drastic reduction in viability or yield of barnase activity.

Attempts to make particular substitutions at two sites produced anomalous but suggestive results. Mutation of the active-site His102 to either lysine or arginine, or of Arg59 to glutamic acid, produced transformants in good numbers, but all such colonies were very small until (within a day or 2) they were overgrown from one or more focus within each. Most of these colonies scored as positive for the mutation when probed with the mutagenic oligonucleotide, but restriction analysis of plasmids prepared from some of them revealed rearrangements or deletions. None of the colonies produced measurable ribonuclease activity. Nevertheless, it is suggested that each of these mutants produces a barnase with a ribonuclease activity insufficiently inhibited by barstar and thereby limiting growth. The R59E mutation could be readily coupled with the inactivating mutation H102G and the inactive

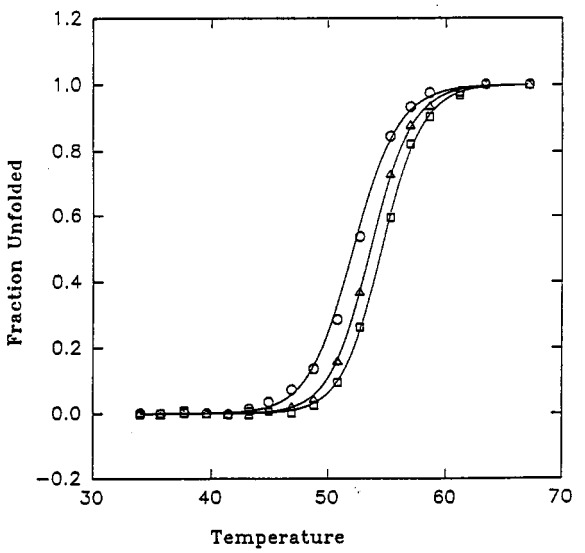

FIGURE 3: Thermal melting curves of (○) barnase, (△) barnase-(H102G), and (□) barnase(R59E,H102G). 0.2 M ammonium acetate/1 mM EDTA pH 8.

double-mutant protein produced in good yield. Addition of a large excess (100-fold) of this protein to barstar and substrate had no effect on the inhibition of subsequently added barnase, indicating no, or very weak, complex formation between barstar and the double mutant. More conservative mutations of Arg59 of barnase to lysine or alanine yielded active enzymes that were not so damaging to the bacterium that usable yields of protein could not be obtained. In both cases, however, growth and barnase production were reduced and barnase-negative clones appeared frequently in cultures. Subsequent findings (see below) that the binding of these mutants to barstar is indeed greatly reduced confirm that the pathology is due to reduced protection of the bacterium from the deleterious effects of barnase activity. Again, the same mutations added to an inactivating mutation at His102, this time H102Q, provided high yields of mutant proteins and no pathology.

The R59A and R59K mutations also affect barnase activity in the hydrolysis of polyethenoadenosine phosphate. When either of these mutant enzymes is added to substrate as in the standard assay described above, there is a short delay during which the increasing fluorescence is concave upward before it becomes linear. The slope of the linear portion is proportional to enzyme concentration, however. With the caveat that the assay is therefore slightly different, it can be said that the R59A and R59K enzymes have specific activities of 28% and 41%, respectively, of that of the wild-type.

As both the His102 and Arg59 side chains lie entirely on the surface of the molecule, mutation of neither should be expected to have a drastic effect on stability. As can be seen in Figure 3, the effects of H102G and R59E are in fact both slight, and, as it happens, both produce small increases in melting temperature. Melting curves of the R59A and R59K barnases were indistinguishable from the wild-type (data not shown).

Barstar Mutants. Barstars carrying any of the charge-reversal mutations listed in Table II were good inhibitors as indicated by sharp end points in barnase titrations. Their yields, however, varied from a few hundred microgram equivalents per liter to the 100–500 mg/L produced by the wild-type. The only barstar mutants found to produce no detectable activity were the double-mutant K21N,K22N, which also produced no antigen, and the two single-mutants D35K and D39K. The last two produce normal amounts of

Table II: Barstar Change-of-Charge Mutants Which Produce Functional Barstar

amino acid substitutions	coding sequence	
	wild-type	mutant
K1N	AAA	AAC
E8K	GAA	AAG
D15K	GAC	AAA
K21Q	AAA	CAG
K22E,E23K	AAGGAG	GAGAAG
E28K	GAA	AAG
E32K	GAA	AAG
E46K	GAG	AAA
E52K	GAA	AAG
R54S	AGG	TCC
E57K	GAA	AAG
K60E	AAG	GAA
E64K	GAA	AAG
E68K	GAG	AAA
R75E,E76R	CGTGAA	GAGCGA
K78E,E80K	AAAGCGGAA	GAAGCGAAA
D83K	GAC	AAG

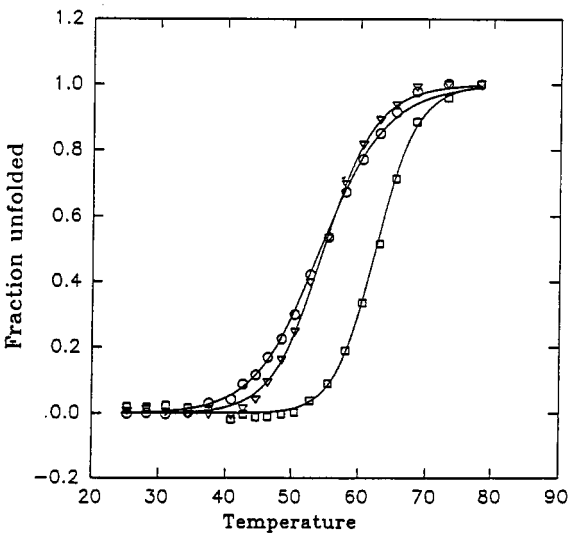

FIGURE 4: Thermal melting curves for (□) barstar, (▽) barstar plus 0.01 M dithiotreitol, and (○) barstar(C40,82A). As Figure 3 plus 3 M urea.

barstar antigen, however, and hence Asp35 and -39 are prime candidates for involvement with the barnase surface.

To investigate the role of the two cysteine residues in barstar, each was mutated to glycine, serine, and alanine, and the three double-mutants C40,82G, C40,82S, and C40,82A were produced. All are functional inhibitors, but yields of the serine and especially the glycine mutants were sharply reduced, with that of the double-glycine derivative being barely detectable. Yields of barstar activity from each of the three cysteine to alanine mutants, however, were comparable to that of the wild-type. The thermal unfolding curve of the double-mutant barstar(C40,82A) (Figure 4) yields a melting temperature (in 3 M urea) about 8 °C below that of the wild-type. When dithiothreitol, as in this case, also shown in Figure 4, or β-mercaptoethanol is included with the wild-type protein, its T_m is reduced to that of the mutant, which is not so affected. This suggests stabilization of the wild-type by a disulfide bond between the two cysteines. The melting curves of the two single Cys to Ala mutants were not significantly different from that of the double mutant. It should be noted also that the low yields of the cysteine to glycine or serine mutants were not reflected in lower melting temperatures *in vitro*. When the structural gene for barstar in pMT702 was replaced by that with the double C40,82A mutation, the resulting clones

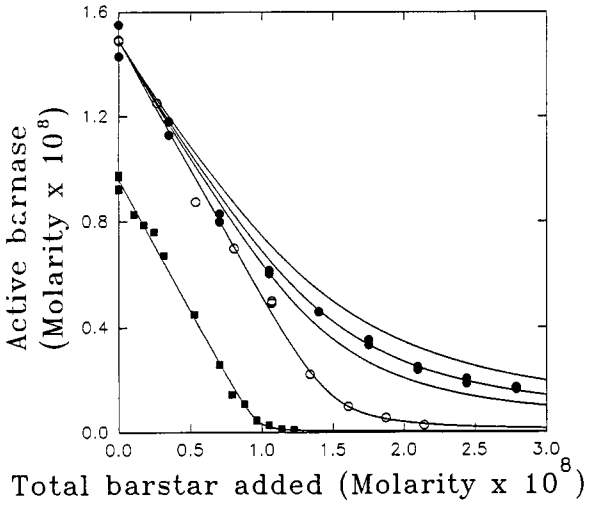

FIGURE 5: Barnase–barstar titrations. (○) Barnase(R59A), barstar(wt); (●) barnase(R59A), barstar(C40,82A); and (■) barnase(R59K), barstar(C40,82A). The smooth curves are based on eq 3 of the text with K_d values (from left to right) of 2.5×10^{-11}, 1.5×10^{-10}, 1.55×10^{-9}, 1.72×10^{-9}, and 1.89×10^{-9} M.

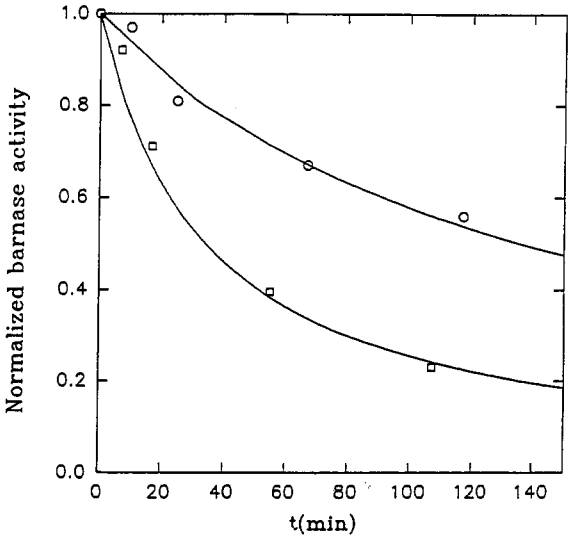

FIGURE 6: Time course of competition for barstar between wild-type barnase and barnase(H102Q). Wild-type barnase added last (at $t = 0$). Initial (=total) wild-type barnase concentration, 4.0×10^{-7} M. Barstar, 4.2×10^{-7} M. Barnase(H102Q): (○) 2.8×10^{-5} M and (□) 7.0×10^{-6} M. The curves are both drawn to eq 2 with $k_{off} = 8.6 \times 10^{-3}$ s^{-1}.

were viable and produced normal yields of barnase activity. This mutant is, therefore, functional *in vivo* as well as *in vitro*.

Protein Sequences. Dideoxy sequencing of the genes for all the barnase and barstar proteins used in binding studies found all sequences as predicted except for the previously noted barnase(H18R) mutation in all derivatives of pMT702.

Barnase–Barstar Binding. For three combinations of active barnase and barstar mutants, binding was weak enough to produce curvature of the titration curve (Figure 5). Molar barstar concentrations are based on titrations of wild-type barnase. As the yields of these two Arg59 barnase mutants were low, their purity was not adequate to allow accurate concentration to be obtained via extinction coefficients. The barnase(R59K) could be titrated accurately with barnase-standardized barstar, and the concentration of barnase(R59A) could be closely estimated by setting the initial slope of its titration cu.ve to −1. These experiments, as noted above, yielded specific activities for the R59K and R59A barnase mutants of 41% and 28%, respectively, of wild-type barnase. The absolute dissociation constants derived from fitting curves to these data are 1.5×10^{-10} M for barnase(R59A) plus wild-type barstar, 1.7×10^{-9} M for barnase(R59A) plus barstar(C40,82A), and 2.5×10^{-11} M for barnase(R59K) plus barstar(C40,82A). The smooth curves in Figure 5, based on eq 3, include curves for one pair with values of K_d 10% above and 10% below that giving a good fit.

Figure 6 shows the results of adding barnase to mixtures of barstar and excess barnase(H102Q). Barnase was added at $t = 0$, and samples were assayed at intervals thereafter. It is clear from these results that barstar binds much more strongly to wild-type barnase than to the mutant. As the complex of barstar with barnase(H102Q) dissociates, the barstar quickly returns to a complex with one of the barnases. Free barnase is measured as activity, here expressed as a fraction of the total free and complexed. As its complex with the wild-type is much more stable, barnase activity declines in time to a very low value.

It is possible to derive a theoretical curve for this decline and obtain an off rate for dissociation of the barstar–mutant barnase complex if we make two assumptions. (1) The on rate constants for complex formation are very high compared to those for the off rates and are the same for both wild-type and mutant barnase. The first half of this assumption is justified by experiment. If barnase is added to premixed barstar and substrate, no activity is observed, indicating very rapid complex formation. Differences in the two on rate constants would not affect the shape of the fitted curves but would introduce error into the derived off rate. (2) The off rate constant (and hence the dissociation constant) for the complex with the mutant barnase is much greater than that with wild-type. This assumption is justified by the measurements themselves. Again, if the on rate constants are all the same, then the dissociation constants are proportional to the off rate constants.

In a mix containing equal total amounts of barstar and wild-type barnase and a large excess of inactive mutant barnase, the concentration and rate of change of free barnase must equal those of the barstar–mutant barnase complex. This rate, in turn, is equal to the rate at which barstar is leaving that complex minus the rate at which it is returning. The latter rate is equal to the former times the fraction of the total free barnase molecules represented by the mutant.

Thus:

$$d[b]/dt = -k_{off}[b] + k_{off}[b](N[b]_0 - [b])/N[b]_0 = -k_{off}[b]^2/N[b]_0 \quad (5)$$

where [b] and $[b]_0$ are the concentrations of free and total active barnase, respectively, k_{off} is the rate constant for dissociation of the barstar–mutant barnase complex, and $N(\gg 1)$ is the ratio of total inactive to total active barnase. This is readily integrated to give, with our starting condition $[b] = [b]_0$ at $t = 0$:

$$[b]/[b]_0 = (N/k_{off})/(t + N/k_{off}) \quad (6)$$

The smooth curves shown in Figure 6 were fitted to the data for both experiments ($N = 17.5$ and $N = 70$) with eq 6 and the same value of $k_{off} = 8.6 \times 10^{-3}$ M^{-1} s^{-1}. Table III shows the values of k_{off} obtained in this manner for various combinations of wild-type and mutant proteins.

The results of several competition equilibrium measurements are given in Table IV. By combining the absolute dissociation coefficients from the titration experiments with the data of Tables III and IV, we can derive rough values for the other

Table III: Rate Constants for Barnase–Barstar Dissociation from Kinetic Competition Experiments[a]

barnase	barstar	k_{off} (s^{-1})
H102D	wt	0.009
H102Q	wt	0.009
H102A	wt	0.5
H102L	wt	1.7
H102Q,R59K	wt	0.5
H102Q,R59A	wt	25
H102D	C40,82A	0.3
H102Q	C40,82A	0.15

[a] Determined by fitting the data to eq 6 of the text.

Table IV: Results of Equilibrium Experiments with both Active and Inactive Barnases in Competition for Barstar

barstar	inactive barnase (b⁻)	active barnase (b)	$[b^-]/[b]$[b]	R_K[a]
wt	H102D	wt	100	3.2×10^{-4}
C40,82A	H102D	wt	100	3.8×10^{-5}
wt	H102Q	wt	100	4.4×10^{-4}
C40,82A	H102Q	wt	100	1.2×10^{-4}
wt	H102G	wt	1000	1.6×10^{-5}
C40,82A	H102G	wt	1000	4.8×10^{-5}
wt	H102Q	R59A	15	1.5
C40,82A	H102Q	R59A	7.5	1.0
wt	H102Q	R59K	6	0.03
C40,82A	H102Q	R59K	75	0.04
wt	H102G	R59A	50	0.03

[a] R_K is the ratio of the dissociation constant of the complex of the barstar with the active barnase to that of its complex with the inactive barnase. See eq 4 in the text. [b] $[b^-]/[b]$ = molar ratio of the total amounts of inactive and active barnase.

Table V: Dissociation Coefficients of Barnase–Barstar Complexes[a]

barnase	barstar	K_d (M)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
wild-type	wild-type	6×10^{-14}	18.0	0.0
wild-type	C40,82A	2×10^{-13}	17.3	–0.7
R59K	wild-type	4×10^{-12}	15.6	–2.4
R59K	C40,82A	2.5×10^{-11} [b]	14.3	–3.7
R59A	wild-type	1.5×10^{-10} [b]	13.3	–4.7
R59A	C40,82A	1.7×10^{-0} [b]	12.0	–6.0
H102Q	wild-type	1.3×10^{-10}	13.5	–4.5
H102Q	C40,82A	1.7×10^{-9}	12.0	–6.0
H102D	wild-type	1.3×10^{-10}	13.5	–4.5
H102G	wild-type	6×10^{-9}	11.2	–6.8
H102A	wild-type	7×10^{-9}	11.1	–6.9
H102L	wild-type	2.5×10^{-8}	10.4	–7.6
H102Q,R59K	wild-type	7×10^{-9}	11.1	–6.9
H102Q,R59A	wild-type	4×10^{-7}	8.7	–9.3

[a] Derived from data of Tables III and IV with the assumption that K_d is proportional to k_{off}. [b] These three values were obtained directly by barstar titration of barnase.

barnase–barstar pairs studied (Table V) including a value of about 10^{-13} for the two wild-type proteins. The free energy, ΔG, for each dissociation is derived from $\Delta G = -RT \ln K_d$.

In Tables III, IV, and V, the barnase mutants R59A, R59K, H102D, H102Q, H102A, and H102L as well as the double-mutants R59K,H102Q and R59A,H102Q all carry the H18R mutation. As barnase(H18R) has been found indistinguishable from the wild-type in equilibrium competition with inactive barnases for barstar, it seems safe to ignore this complication.

DISCUSSION

In the course of this mutagenic survey, I have identified four residues which are clearly involved in the binding of barnase to barstar. Several mutations of two residues of barnase, Arg59 and His102, both of which are also involved in its enzymatic activity, have been studied in some detail. Mutation of the two barstar residues, Asp35 and Asp39, shows that they are involved in barnase recognition but their mutants have not been characterized beyond their failure to inhibit. In addition, mutation of the two cysteines of barstar to alanines produces a small but definite decrease in the binding energy. From the three-dimensional structure of the double mutant in the complex, it seems likely that this effect is due to subtle changes in the barstar binding site related to a conformational change required by the substitutions. Barnases with substitutions at either His102 or Arg59 appear to discriminate between wild-type barstar and the C40,82A mutant better than does wild-type barnase.

The changes in the free energy of binding ($\Delta\Delta G$) for the mutations of residues Arg59 and His102 of barnase are additive, or roughly so, implying that their interactions with barstar are independent. In the crystallographic structure, they do clearly interact with different residues of barstar. As might be expected, the effects of changing Arg59 to Lys, Ala, and Glu increase in that order, with the R59E mutant being insufficiently inhibited for survival of clones even with the low level of barnase present without induction. For the His102 mutants, it somewhat surprising to find that the aspartic acid derivative binds barstar as well as H102Q and better than H102G or H102A.

By combining the dissociation coefficients derived from barnase–barstar titration and competitive equilibrium measurements with measured rate constants for dissociation, we can compute rate constants for association of about 10^8 M^{-1} s^{-1}. This is on the order of rates reported for insulin dimerization (Koren & Hammes, 1976) and within the range expected for the diffusion-controlled association of molecules with the size and charge differences of barnase and barstar (Northrup & Erickson, 1992). Combining this on rate constant with a K_d of 10^{-13} M gives us an estimate of 10^{-5} s^{-1} for the off rate constant of the wild-type complex.

Of other enzyme plus protein inhibitor systems, only a number of protease–inhibitor pairs, recently reviewed by Bode and Huber (1992), are understood in any structural detail, and these systems promise a great deal of information on protein folding and protein–protein recognition. The inhibitors in these cases include, however, a natural substrate for the protease in the form of a scissile peptide bond. A ribonuclease inhibitor does not have this option, and barstar maintains its covalent integrity through inhibition and its reversal, an advantage when one studies the kinetics and equilibrium of the complex.

Continuation of this work will include analysis by the procedures developed here of the two aspartic acids at positions 35 and 39 of barstar as well as other residues in the interface as seen in the new structure. It will require more detailed analysis of the structure of the complex and, probably, solution of more structures before we can provide any quantitative explanations for results such as those reported here. In the future, NMR solution of the barnase–barstar structure should provide us with a rapid method of analyzing the structural rearrangements required by amino acid substitutions in the barnase–barstar interface. General conclusions on the relation between fit and free energy in the interface should be applicable to such relations within, as well as between, domains.

ACKNOWLEDGMENT

I thank Dr. Yelena Chernokalskaya for sequencing of the mutant genes.

REFERENCES

Baudet, S., & Janin, J. (1991) *J. Mol. Biol. 219*, 123.

Bode, W., & Huber, R. (1992) *Eur. J. Biochem. 204*, 433.

Bycroft, M., Ludvigsen, S., Fersht, A. R., & Poulsen, F. M. (1991) *Biochemistry 30*, 8697.

Hartley, R. W. (1975) *Biochemistry 14*, 2367.

Hartley, R. W. (1980) *J. Mol. Evol. 15*, 355.

Hartley, R. W. (1988) *J. Mol. Biol. 202*, 913.

Hartley, R. W. (1989) *Trends Biochem. Sci. 14*, 450.

Hartley, R. W., & Rogerson, D. L. (1972) *Prep. Biochem. 2*, 229.

Hartley, R. W., & Smeaton, J. R. (1973) *J. Biol. Chem. 248*, 5624.

Hartley, R. W., & Fitzgerald, P. C. (1989) in Proceedings of the First International Meeting on the Structure and Function of Ribonucleases, Nov 28–Dec 2, 1988 (Pavlovsky, A., & Polyakov, K., Eds.) pp 322–327, Moscow.

Hill, C., Dodson, G., Heinemann, U., Saenger, W., Mitsui, Y., Nakamura, K., Borisov, S., Tischenko, G., Polyakov, K., & Pavlovsky, S. (1983) *Trends Biochem. Sci. 8*, 1.

Jones, D. H., & Howard, B. H. (1990) *BioTechniques 8*, 178.

Koren, R., & Hammes, G. G. (1976) *Biochemistry 15*, 1165.

Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C., & Jack, A. (1982) *Nature 297*, 162.

Northrup, S. H., & Erickson, H. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3338.

Pace, C. N., Laurents, D. V., & Erickson, R. E. (1992) *Biochemistry 31*, 2728.

Paddon, C. J., & Hartley, R. W. (1987) *Gene 53, 11.*

Prevost, M., Wodak, S. J., Tidor, B., & Karplus, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10880.

Reynolds, J. A., & Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002.

Serrano, L., Matouschek, A., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 847.

Speyer, J. F. (1990) *BioTechniques 8*, 548.