Stability and function: two constraints in the evolution of barstar and other proteins

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Background: Barstar is the intracellular inhibitor of barnase, an extracellular RNAse of *Bacillus amyloliquefaciens*. The dissociation constant of the barnase–barstar complex is 10^{-14} M with an association rate constant between barnase and barstar of 3.7×10^{8} s^{-1} M^{-1}. The rapid association arises in part from the clustering of four acidic residues (Asp35, Asp39, Glu76 and Glu80) on the barnase-binding surface of barstar. The negatively charged barnase-binding surface of barstar effectively 'steers' the inhibitor towards the positively charged active site of barnase.

Results: Mutating any one of the four acidic side chains of barstar to an alanine results in an approximately two-fold decrease in the association rate constant, while the dissociation rate constant increases from five orders of magnitude for Asp39→Ala, to no significant change for Glu80→Ala. The stability of barstar is increased by all four mutations, the increase ranging from 0.3 kcal mol^{-1} for Asp35→Ala or Asp39→Ala, to 2.1 kcal mol^{-1} for Glu80→Ala.

Conclusions: The evolutionary pressure on barstar for rapid binding of barnase is so strong that glutamate is preferred over alanine at position 80, even though it does not directly interact with barnase in the complex and significantly destabilizes the inhibitor structure. This, and other examples from the literature, suggest that proteins evolve primarily to optimize their function *in vivo*, with relatively little evolutionary pressure to increase stability above a certain threshold, thus allowing greater latitude in the evolution of enzyme activity.

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Introduction

There is a continuous evolutionary pressure for changes in protein sequences, and conserved regions are those that resist this pressure. The most conserved regions of sequence in a family of homologous proteins are usually found in the active site, especially the residues directly involved in activity. Conversely, residues outside active sites are much less conserved, especially those on the surface of a protein [1–3]. There are thus many ways of achieving the same tertiary structure without affecting the stability of a protein, whereas a restricted set of residues is required for the optimization of a specific activity. This poses a question: what is the compromise between the optimization of structural stability and the optimization of activity in the evolution of proteins?

Both stability and activity of proteins *in vitro* can be increased substantially [4–13]. Comparisons of homologous proteins from thermophiles and mesophiles have shown that related proteins can perform the same function, yet have very different stabilities [4–6]. Protein stability can also be increased by site-directed mutagenesis, without affecting the activity of the protein. Examples of this include the results of deleting the salt bridge between Asp12 and Arg110 in barnase [7], of introducing mutations to alanine in a helix of barnase and T4 lysozyme [8,9], and the results of the multiple mutations which convert barnase to binase, its very close homologue from *Bacillus intermedius*, and which increase stability by up to 3.3 kcal mol^{-1} [10]. Increased stability of barnase was also achieved by mutation of positively charged residues in the active site, leading to reduced activity [11]. Increased protein activity achieved by site-directed mutagenesis was shown, for example, for insulin [12] and for human growth hormone binding its receptor [13].

Barstar is the intracellular inhibitor of barnase, an extracellular RNAse of *Bacillus amyloliquefaciens*. Barstar is necessary for survival of barnase-producing cells, since intracellular barnase activity is lethal to the organism. Barstar consists of a single chain of 89 amino acids, of M_r 10 211 [14]. The solution structure of free barstar has been solved by NMR [15], while the crystal structure of a barnase–barstar complex has been solved to high resolution [16,17]. Barstar is composed of three parallel α-helices packed against a three-stranded β-sheet, with a short fourth helix serving as a cap. The ionic residues in the barnase-binding site of barstar are exclusively negatively charged, with four acidic residues on the barstar surface; Asp35 and Asp39 are located on α-$helix_2$ (which partly blocks the barnase active site cleft in the complex). Asp39 is especially important in the interaction with barnase since it makes hydrogen bonds with Arg83, Arg87, and the catalytic histidine (residue 102) of barnase. Asp39 effectively mimics the reactive phosphate group of an RNA substrate [16,17]. Glu76 and Glu80 are both located on α-$helix_4$ of barstar. Glu76 forms a salt bridge with Arg59 of barnase. Although Glu80 does not directly interact with barnase in the complex, it is 7 Å from the barnase surface and makes indirect, water-mediated, hydrogen bonds with barnase (Fig. 1). There is

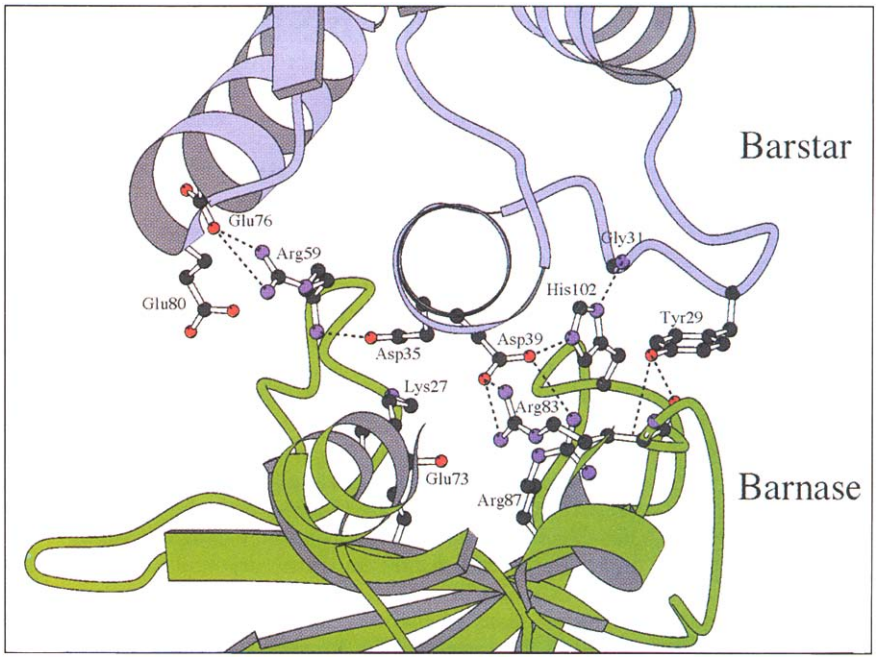

Fig. 1. Cross-section through the barnase–barstar interface, showing some important protein–protein interactions and the residues mutated in this study. Hydrogen bonds are drawn as broken lines. This figure was drawn with the MOLSCRIPT program [32].

a predominance of positively charged over negatively charged residues (Lys27, Arg59, Arg83 and Arg87 *versus* Asp54, Glu60 and Glu73) in the active site of barnase [11,17]. The highly electrostatic nature of the barnase–barstar interaction results in a very high association rate constant of 3.7×10^8 s^{-1} M^{-1}, which is about 100 times faster than usually observed for the association of two protein molecules [18,19]. The association rate constant (subsequently referred to as the 'on rate') can be increased by removing any one of the three acidic residues in the barstar-binding site of barnase, whereas removal of any basic active-site residues of barnase reduces the on rate by up to 10-fold. Since the association rate constant is strongly dependent on salt concentration, it is clear that electrostatic forces are very important for the association of these two molecules [18].

The aim of this work is to investigate, using site-directed mutagenesis, the apparent compromise between barstar stability and activity.

Results

The negatively charged binding surface of barstar has an important role in barnase–barstar association

The barnase-binding surface of barstar comprises four negatively charged residues (Asp35, Asp39, Glu76 and Glu80), with no positively charged residues on this face of the protein structure (Fig. 2). Mutating any one of these acidic residues to an alanine decreases the association rate constant by about two-fold, with no significant variation among the four mutations (Table 1). In contrast, removal of positively charged side chains from the active site of barnase results in a decrease in the association rate constant by a factor of 2–10 [18]. These in on rates. However, Arg83 and Arg87 are partially buried in a small cleft in the surface of the active site of barnase, while Lys27 and Arg59 are fully exposed, resulting in the latter having an increased influence on the on rate reaction.

Dissociation rate constants for barstar mutations vary by up to five orders of magnitude compared with wild-type: the Asp39→Ala mutation results in a dissociation rate constant of 0.9 s^{-1}, compared with 3.7×10^{-6} s^{-1} for wild-type barstar, corresponding to a change in the free energy of binding ($\Delta\Delta G$) of 7.7 kcal mol^{-1} (Table 1). This large decrease in binding energy can be explained by the many salt bridges and hydrogen bonds between Asp39 and barnase active-site residues Arg83, Arg87 and His102 (Fig. 1); the Asp35→Ala mutation increases the dissociation rate constant (off rate) by three orders of magnitude to 0.0038 s^{-1} ($\Delta\Delta G$=4.5 kcal mol^{-1}). The side chain of Asp35 makes hydrogen bonds with the barnase Arg59 backbone NH, and also makes van der Waals interactions with the arginine side chain; the Glu76→Ala mutation increases the off rate by a factor of six to 2.1×10^{-5} ($\Delta\Delta G$=1.4 kcal mol^{-1}). This loss in binding energy probably results from the removal of a salt bridge between Glu76 in barstar and Arg59 in barnase. The mutation Glu80→Ala does not significantly effect the dissociation rate constant, since Glu80 does not directly interact with barnase.

The stability of barstar is increased by mutating any one of the four acidic residues on the barnase-binding site

The free energy of unfolding in water ($\Delta G_{U-F}^{H_2O}$) was measured as a function of the change in barstar fluorescence upon titration with urea. The entire data set was fitted to a two-state unfolding transition curve as de-

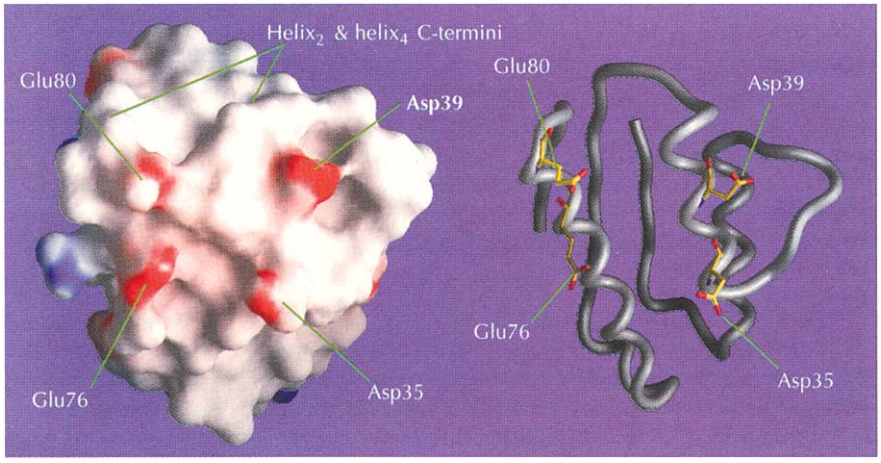

Fig. 2. Representations of the structure of barstar, showing the barnase-binding surface and locations of the residues mutated in this study. Left; molecular surface of barstar colour coded according to electrostatic potential (calculated by GRASP [33]). Positively charged regions are coloured blue, negatively charged regions red. Right; backbone of barstar, drawn in the same orientation.

Table 1. Association (k_1) and dissociation (k_{-1}) rate constants of barnase with wild-type and mutant barstar at pH 8.[a]

Barstar	$k_1 \times 10^{-8}$ ($s^{-1}\,M^{-1}$)	$k_{-1} \times 10^{6}$ (s^{-1})	K_i(pM)	ΔG^b (kcal mol^{-1})	$\Delta\Delta G^c$ (kcal mol^{-1})
Wild-type	3.7	3.7	0.01	−19.0	
Asp35→Ala	1.9	3800	20	−14.5	−4.5
Asp39→Ala	1.9	900 000	4100	−11.3	−7.7
Glu76→Ala	2.0	21	0.1	−17.6	−1.4
Glu80→Ala	2.0	5.2	0.025	−18.5	−0.5

[a]All rate constants were measured in 50 mM Tris-HCl buffer at 25 °C. K_i was calculated from the equation $K_i = k_1/k_{-1}$. [b]The free energy of binding was calculated from: $\Delta G = -RT\ln K_i$. [c]The difference in free energy of binding wild-type and mutant proteins $\Delta\Delta G = \Delta G_{wt} - \Delta G_{mutant}$. Standard errors for association and dissociation rate constants are ± 15%, which results in an error in ΔG of ± 0.11 kcal mol^{-1}.

Table 2. Changes in the free energies of unfolding of wild-type barstar and mutant proteins.[a]

Barstar	$[urea]_{50\%}$ (M)	$\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})[b]	$\Delta\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})[c]
Wild type	4.19	−5.28	0
Asp35→Ala	4.45	−5.60	0.3
Asp39→Ala	4.42	−5.57	0.3
Glu76→Ala	4.84	−6.09	0.8
Glu80→Ala	5.90	−7.42	2.1

[a]The free energy of unfolding was determined by fluorescence changes on denaturation with urea at 25°C, 50 mM Tris-HCl pH 8, 10 mM DTT [20]. [b]The free energy of unfolding in water was determined by multiplying the average value of m (1.26 kcal mol^{-2}) by $[urea]_{50\%}$. [c]The difference in free energy of unfolding wild-type and mutant proteins $\Delta\Delta G_{U-F} = \Delta G_{wt} - \Delta G_{mutant}$. Standard errors for $[urea]_{50\%}$ are of a magnitude of ± 0.06 M urea, which corresponds to a free energy (ΔG) of ± 0.075 kcal mol^{-1}.

results in an increase in the stability of the folded protein (Table 2). The largest increases in stability were found for mutations Glu76→Ala (0.8 kcal mol^{-1}) and Glu80→Ala (2.1 kcal mol^{-1}). Deletion of the Glu80 side chain increases the total stability of the protein by about 40%. In contrast, mutation of either Asp35 or Asp39 increases the overall stability by only 0.3 kcal mol^{-1}. None of the mutations affect the overall structure of the protein, as monitored by far UV circular dichroism (data not shown).

The increased stability of barstar mutants has an electrostatic component

The addition of 300 mM NaCl increases the stability of wild-type barstar by 0.6 kcal mol^{-1} (Table 3). We interpret this as resulting from increased electrostatic screening between the four clustered acidic side chains. Under these conditions, the increase in the free energy of unfolding for the mutant proteins, relative to wild-type, is 0.05 kcal mol^{-1} for Asp35→Ala, 0.1 kcal mol^{-1} for Asp39→Ala, 0.6 kcal mol^{-1} for Glu76→Ala and 1.4 kcal mol^{-1} for Glu80→Ala. A comparison between the change in free energy of unfolding for the different mutant proteins relative to wild-type, with and without the addition of NaCl, shows that the greater the contribution stability on the ionic strength of the solution. This is further evidence to support the argument that electrostatic repulsion makes a major contribution to the inherent instability of the wild-type structure. The Glu80→Ala mutation is an extreme case, where the addition of 300 mM salt actually destabilizes the structure by 0.1 kcal mol^{-1}.

Increasing the ionic strength of the solution from 300 mM to 700 mM NaCl increases the stability of wild-type barstar by an additional 1.15 kcal mol^{-1}. The increased salt concentration does not significantly alter the relative free energy of mutant *versus* wild-type unfolding ($\Delta\Delta G^{H_2O}_{U-F}$) for the mutants Asp35→Ala, Asp39→Ala, and Glu76→Ala. However, the mutation Glu80→Ala causes a further decrease of 0.3 kcal mol^{-1} in the free energy of unfolding, relative to wild-type.

Discussion

We have investigated the compromise between structural stability and activity in the evolution of barstar. In any such study, the requirements of the protein *in vivo* must

Table 3. Changes in the free energies of unfolding of wild-type barstar and mutant proteins in the presence of 300 mM and 700 mM NaCl.[a]

Barstar	300 mM NaCl				700 mM NaCl			
	$[urea]_{50\%}$ (M)	$\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})	$\Delta\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})	$\Delta\Delta G_{0-300}$ mM NaCl[b]	$[urea]_{50\%}$ (M)	$\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})	$\Delta\Delta G^{H_2O}_{U-F}$ (kcal mol^{-1})	$\Delta\Delta G_{0-700}$ mM NaCl[b]
Wild-type	4.67	−5.88	0	0.6	5.58	−7.03	0	1.8
Asp35→Ala	4.71	−5.93	0.1	0.3	5.63	−7.09	0.1	1.5
Asp39→Ala	4.77	−6.01	0.1	0.4	5.76	−7.26	0.2	1.7
Glu76→Ala	5.11	−6.44	0.6	0.4	5.99	−7.55	0.5	1.5
Glu80→Ala	5.8	−7.30	1.4	−0.1	6.45	−8.13	1.1	0.7

[a]The free energy of unfolding was determined by fluorescence changes on denaturation with urea at 25°C, 50 mM Tris-HCl pH 8, 10 mM DTT + 300 mM or 700 mM NaCl as for Table 2. [b]The difference in free energy of unfolding in the presence of NaCl.

increased as well, and, if it does, such an increase might not be favourable for the system as a whole. The problem is simplified when studying a protein with a specific function that can be increased without affecting other cellular functions. The only known role of barstar is to be the intracellular inhibitor of barnase. Further, when analyzing the effects of single mutations in a protein, we are just looking at the fine-tuning of the protein to its environment and role.

Barstar is necessary for survival of barnase-producing cells, since intracellular barnase activity would be lethal to the organism. As a consequence, barstar is required to bind rapidly and tightly to any intracellular, active barnase molecules. The strongly negatively charged binding surface of barstar, effectively 'guides' the protein during its association with the positively charged barnase active site [17,18]. A mutation of any one of these acidic side chains of barstar decreases the association rate constant by a factor of about two. We have shown that barstar achieves this very fast binding at the expense of its stability. The free energy of folding of reduced wild-type barstar is around 5 kcal mol^{-1}, which is at the lower end of the range of stabilities measured for other small globular proteins [21]. Clusters of like-charged residues on the surface of proteins have been shown previously to have a destabilizing effect on protein stability [22]. For example, the repulsion energy between two aspartic acid side chains in α-$helix_1$ of barnase (Asp8 and Asp12) was estimated to be 0.3 kcal mol^{-1} [7]. Mutations of Lys27 and Arg59 in the positively charged barnase active site increases the stability of the protein by 0.36 kcal mol^{-1} and 0.64 kcal mol^{-1}, respectively [11]. A similar repulsion energy was measured for a charged His–Lys pair in subtilisin [23]. Barstar residues Asp35 and Asp39 are located on the same helix, and their side chain carboxyl groups, which are well defined, are separated by about 6 Å in the NMR solution structure of the reduced, free wild-type protein [15]. Mutating Asp35 or Asp39 to alanine increases the free energy of unfolding by about 0.3 kcal mol^{-1}. The addition of salt diminishes most of this increase in stability, suggesting some electrostatic repulsion between these two residues. Glu76 and Glu80 are located on α-$helix_4$ of barstar. The increase in free energy of unfolding upon mutating these two residues to alanine was found to be 0.8 kcal mol^{-1} and 2.1 kcal mol^{-1}, respectively. The dependence of $\Delta\Delta G^{H_2O}_{U-F}$ on salt concentration shows that there is also some electrostatic repulsion between these two residues. Since the presence of alanine mutations at residues 76 and 80 also causes increases in stability of 0.5 kcal mol^{-1} and 1.1 kcal mol^{-1}, respectively, even in the presence of 700 mM NaCl, we cannot exclude the possibility that some factor other than electrostatic repulsion might also be contributing to the inherent instability in this region of the wild-type structure, for example the difference in helix-forming tendencies between alanine, glutamic acid and aspartic acid [24]. The intracellular ionic strength in bacteria depends on the osmolarity of the growth medium, and was found to be at around 200 mM for osmolarities up to 0.2 osM in *Escherichia coli* cells [25]. The instability due to glutamic acid at positions 76 and 80 is still very significant at this ionic strength.

The presence of a glutamic acid residue at position 80, rather than an alanine, shows to what extent in evolution stability will be sacrificed for activity. A glutamic acid at this position has no substantial effect on the dissociation rate constant of the barnase–barstar complex, and increases the association rate constant by a factor of only two. Yet, the protein is destabilized by 2.1 kcal mol^{-1} in the presence of this negatively charged residue, relative to an alanine in the mutant. To our knowledge, the increase in stability due to this mutation is larger than any other in the literature. Smaller values have been found for single mutants in barnase, T4 lysozyme and insulin [9,10,12]. Our results suggest that rapid, and not just tight, binding are the predominant factors in the evolution of barstar.

Is there a structural basis for the measured differences in $\Delta\Delta G^{H_2O}_{U-F}$ of unfolding for the four barstar mutations? We find that mutations at positions 35 and 39 stabilize the protein by only 0.3 kcal mol^{-1}, yet mutations at positions 76 and 80 increase stability by 0.8 kcal mol^{-1} and

near to the carboxyl termini of $helix_2$ and $helix_4$, there could be an unfavourable electrostatic interaction between the side chain and the helix macro-dipoles. Fig. 2 shows that Glu80 could be sterically restricted by the repulsion between this side chain and the negative charges at positions 35, 39, 76, and the carboxyl termini of α-helices 2 and 4. This could explain why the Glu80 side chain is well defined in the NMR structure (with a similar orientation as in the crystal structure of the complex). It is interesting to note that the side chains of Asp35 and Glu76 are also well defined in the NMR solution structure, and show similar orientations in the free and complexed structures [15,17] (the Asp39 side chain is not well defined in the NMR structure). This pre-ordering of side chain conformations will be entropically unfavourable, and will result in destabilization of the protein. On the other hand, it will contribute to the stability of the complex, since the entropic loss upon formation of the complex will be lowered. Secondly, the Glu80 mutation could remove a favourable interaction in the unfolded state, thereby stabilizing the folded structure. This is unlikely, however, since there is sufficient evidence from NMR studies that barstar has little residual unfolded structure (MJ Lubienski and M Bycroft, personal communication).

Analysis of activity *versus* stability in the barnase active site, using site-directed mutagenesis, gave similar results to those of barstar, although stability is compromised to a lesser extent and activity losses due to mutations are much greater than found in barstar [11]. Also, the example of barnase is more complicated since barnase has evolved to bind both barstar and RNA, and so any compromise between structure and activity will reflect this. We can also ask the question: if the active site of barnase has small areas of negative charge (Asp54, Glu73, and Glu60 — the last two are important in the barnase–RNA substrate interaction [26]), why does barstar not have any complementary positively charged regions on its binding surface? One explanation is that the many electrostatic interactions between barnase and barstar result in a complex of sufficiently high stability, and that in barstar evolution, there existed further pressure for rapid binding (rather than only tight binding). This was achieved by simply increasing the negative charge on the binding site.

Are our findings general? When increased stability is of major importance, as in thermophilic bacteria, we see that homologous enzymes can have very different stabilities. In the mapping of stability *versus* activity of bacterial tyrosyl-tRNA synthetase from *E. coli versus Bacillus stearothermophilus* it was possible to improve significantly the activity of the thermophilic enzyme by constructing chimeric proteins with the *E. coli* enzyme. But this was achieved at the expense of stability, which, in this case, is crucial in the evolution of the enzyme [6]. However, when increased stability over a certain stability by mutagenesis without affecting activity [9,10]. The best example so far to illustrate this point is in the systematic stepwise evolution of barnase to its very closely related homologue, binase. A chimeric protein containing all the stabilizing changes has a similar activity to both parents, but has a significantly higher stability than either [10].

Biological implications

During evolution, every protein is subjected to a constant pressure for change, resulting from the insertion of random mutations during replication. Despite this, we find a strong tendency for conserving residues related to activity, but not to stability. An implication of this is that activity is highly specified, while there are many possible ways to achieve sufficient stability. In other words, a protein structure has a much higher tolerance for changing structural amino acids than for changing functional ones. In recent years, at least four cases where stability can be greatly increased with no compromise in activity have been identified using site-directed mutagenesis. These suggest that there are many ways to achieve sufficient stability for a protein with a given structure. The problems frequently encountered in engineering an increased stability for a protein probably stem, therefore, from our lack of understanding of the factors that govern protein stability.

Here we report the results of mutating four residues of the nuclease inhibitor barstar that are important in the formation of a complex between barstar and its target nuclease, barnase. These mutations increase the stability of barstar but decrease the reaction rates of complex formation and dissociation. We conclude that barstar has compromised its stability to a large extent to optimize its specific activity *in vivo*. In evolutionary terms, the latitude that a protein has with respect to stability will result in an ability to change its sequence readily, allowing the optimization of enzyme activity. This will also give a protein the freedom needed for adjusting itself to the constant changes in the evolutionary process.

Materials and methods

Protein expression and purification

Site-directed mutagenesis of barstar was performed by the method of Sayers *et al.* [27]. The oligonucleotides used to introduce an alanine codon were: for Asp35; 5′-CCA TAA AGC GGC CAG GTT TTC-3′, for Asp39; 5′-GGT CAG ACA AGC CCA TAA AGC-3′, for Glu76; 5′-GCT TTC GCT GCA CGG AAA-3′, and for Glu80; 5′-GTC GCA GCC TGC CGC TTT CGC-3′. Mutant plasmids were ident-

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