J. Mol. Biol. (1993) **233**, 305–312

Step-wise Mutation of Barnase to Binase

A Procedure for Engineering Increased Stability of Proteins and an Experimental Analysis of the Evolution of Protein Stability

Luis Serrano†, Anthony G. Day and Alan R. Fersht

MRC Unit for Protein Function and Design
Cambridge Centre for Protein Engineering
MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

(Received 9 March 1993; accepted 26 May 1993)

We have chosen two members of the microbial RNase family, barnase and binase, which have 85% identity (17 substitutions and 1 deletion) and almost identical three-dimensional structure, to study the evolution of protein stability. The 17 residues that differ are scattered throughout the molecule. Each of the 17 differing residues has been mutated independently and the effect on protein stability analysed. Each point mutation has an effect on protein stability that ranges from +1·1 to −1·1 kcal mol^{-1}. These changes in energy are additive. There is no clear correlation between the type of mutation and the effect on protein stability. A multiple mutant having six of the single mutations that increase the stability of barnase is 3·3 kcal mol^{-1} more stable than wild type and has the same activity. There could be selective pressure to maintain proteins at a certain stability and, consequently, mutations that decrease stability tend to be counterbalanced by stabilizing mutations. Alternatively, there could simply be pressure to maintain stability above a certain level, and any further increases in stability need not be maintained during evolution. These results suggest a simple way to improve the stability of proteins: choose two homologous proteins that have high similarity, mutate individually all of the residues that differ between the two, and combine the mutations that increase the stability in a multiple mutant.

Keywords: protein stability; protein activity; co-operativity; protein evolution; barnase

1. Introduction

The enhancement of protein stability by rational design is one of the great goals of protein engineering. Two approaches have been followed to alter the stability of proteins. First, the analysis by protein engineering of the contribution of the different interactions that take place in a protein has resulted in some general rules about possible ways to increase the stability of a protein (reviewed by Fersht & Serrano, 1993). Second, the comparison between homologous proteins from thermophiles and mesophiles has provided some insight into the reasons why related proteins performing the same function could have very different stabilities (Argos *et al.*, 1979; Imanaka *et al.*, 1986; Vihinen, 1987; Menedez-Arias & Argos, 1989; Mrabet *et al.*, 1992). A complete analysis of the differences between homologous thermo-stable and normal proteins is, however, difficult, since in general they have diverged significantly. Consequently, it is very likely that there will be slight conformational rearrangements and co-operative effects from those residues that are different (Imanaka *et al.*, 1987). It is important to examine proteins that are at the beginning of divergence, since their structures are still almost identical and the effect of single differences can be easily analysed.

Barnase, a small ribonuclease from *Bacillus amyloliquefaciens*, has proved to be a good paradigm for thermodynamic studies. This protein consists of a single polypeptide chain of 110 amino acid residues. Its three-dimensional structure has been determined by X-ray crystallography (Mauguen *et al.*, 1982; Baudet & Janin, 1991) and by NMR in solution (Bycroft *et al.*, 1991). Barnase has a close relative, binase, whose sequence is identical for all but 18 residues (Hill *et al.*, 1983; Pavlovsky *et al.*, 1983, 1989) and whose three-dimensional structure

† Present address: EMBL Biostructures and Biocomputing Programme, Meyerhofstrasse 1, Heidelberg, Germany.

0022-2836/93/180305-08 $08.00/0

is known (Pavlovsky *et al.*, 1983, 1989). Barnase undergoes reversible denaturation, mediated by denaturants or heat, which closely follows a two-state process (Hartley, 1975; Kellis *et al.*, 1988). Changes in the free energy of unfolding can be accurately measured (Kellis *et al.*, 1988, 1989; Serrano *et al.*, 1990; Horovitz *et al.*, 1992). In this study, we have independently mutated each one of the 17 residues† that differ between the two proteins and have determined the changes in stability. We have also made multiple mutants in order to look at the additivity of changes in stability and have measured the activity of the mutant enzymes.

2. Experimental Procedures

(a) *Materials*

The substrates, GpUp and RNA type XI from bakers' yeast were obtained from Sigma. The buffer used in the denaturation experiments was 2-[*N*-morpholino]ethanesulphonic acid (MES) from Sigma. The MES stock was a 1·00 M solution containing 387 mM acid form and 613 mM sodium salt, which gives a pH of 6·3 at 25°C on dilution to 50 mM. Urea was enzyme grade from Bethesda Research Laboratories, MD, U.S.A. Radiochemicals were from Amersham International, and SP-Trisacryl was obtained from IBF, Villeneuve La Garenne, France. *Escherichia coli* BL21DE3 pLysS was a generous gift from Dr F. W. Studier. Plasmid pTZ18U and the helper phage M13KO7 were obtained from Pharmacia, Sweden. Wild-type barnase in the plasmid pUC19 (Paddon & Hartley, 1987), generously donated by Dr Hartley, was recloned into the pTZ18U plasmid (Serrano & Fersht, 1989; Serrano *et al.*, 1990). The X-ray co-ordinates for barnase were provided by Dr K. Henrick, Professor G. Dodson and A. Cameron. Wild-type binase from *Bacillus intermedius*, was generously provided by Professor M. Ya. Karpeisky. Binase was also expressed from a synthetic gene which was constructed as described in section (b), below.

(b) *Mutagenesis*

Single-stranded DNA was obtained from the modified pTZ18U, harboured in *E. coli* TG2 after infection with the helper phage M13KO7 (Pharmacia), following the conditions described by Pharmacia. Site-directed mutagenesis was carried out using the method of Eckstein (Sayers *et al.*, 1988) and the kit supplied by Amersham (using 1/5 of the recommended amounts of DNA and kit). Mutants were identified by directly sequencing the single-stranded DNA. The efficiencies of mutation ranged from 50 to 99%. The mutations Thr16 → Arg, His18 → Lys, Ile55 → Val and Leu89 → Val have been previously described (Serrano *et al.*, 1990, 1992; Loewenthal *et al.*, 1992). The synthetic binase gene was constructed by repeated inverse polymerase chain reaction (PCR) mutagenesis on the barnase plasmid using 6 pairs of mutagenic primers (Clackson *et al.*, 1991).

(c) *Expression and purification of barnase and synthetic binase*

These were performed as described previously (Serrano *et al.*, 1990).

(d) *Urea denaturation*

The reversible unfolding was monitored by fluorescence spectroscopy with excitation at 290 nm and emission at 315 nm, as described previously (Horovitz *et al.*, 1992).

(e) *Solvent-accessible area calculations and atom distances*

The solvent-accessible area calculations and the distances between atoms were calculated from the surface generated by the locus of the centre of a sphere of radius 1·4 Å rolling over the exposed van der Waals' surface using the program WHAT IF (Vriend, 1990).

(f) *Activity measurements*

These were performed as described previously for the dinucleotide substrate GpUp (Day *et al.*, 1992) and for RNA type XI from bakers' yeast (Mossakowska *et al.*, 1989).

3. Results

(a) *Description of the mutants*

The majority of the substitutions between barnase and binase are conservative, with seven exceptions (Gln15 → Ile, Thr16 → Arg, Gln31 → Ser, Gly65 → Ser, Lys66 → Ala, Thr79 → Val and Gln104 → Ala) and only two involve completely buried residues (Ile88 → Leu and Leu89 → Val)

Table 1

Solvent accessibility of the residues that differ between barnase and binase

Position	Barnase residue accessibility (%)	Binase residue accessibility (%)
15	31	35
16	31	57
18	46	49
19	73	41
29	40	42
31	47	37
44	64	64
55	46	48
62	55	50
65	59	70
66	55	39
79	63	62
85	20	27
88	0	0
89	0	0
104	62	34
108	47	47

The percentage of the accessibilities of the residues in the folded state with respect to the unfolded state in a Gly-X-Gly tripeptide (Miller *et al.*, 1987) are indicated.

† There are 17 amino acid substitutions and a deletion, Gln2 → Δ, on going from barnase to binase. The Gln2 → Δ deletion lies in a region of the protein that is not visible in the crystal structures and that appears disordered in the NMR solution structure (Bycroft *et al.*, 1991). This deletion was not investigated in this work and was not incorporated in the synthetic binase gene that was constructed.

Table 2
Hydrogen bonds made by the side-chains of those residues that are different between barnase and binase

Residue	Donor	Acceptor	Distance (Å)
Gln15 (barnase)	$N^{\varepsilon 2}$	O Trp94	2·5
Thr16 (barnase)	$O^{\gamma 1}$	O Asp12	3·0
Arg19 (binase)	$N^{\eta 2}$	OH Tyr13	2·6
Glu29 (barnase)	$O^{\varepsilon 1}$	N Thr26	2·8
Ser31 (binase)	O^{γ}	O Ser28	2·8
Lys62 (barnase)	N^{ζ}	O Gln104	2·9
Arg62 (binase)	$N^{\eta 1}$	O Ala104	2·9
Gln104 (barnase)	$N^{\varepsilon 2}$	O Asp101	3·1
Lys108 (barnase)	N^{ζ}	O Arg110	2·9
Arg108 (binase)	$N^{\eta 1}$	O Arg110	2·5

(Table 1). In the first α-helix, there are three mutations: Gln15 → Ile, Thr16 → Arg and His18 → Lys. Mutation of Gln15 to Ile results in the deletion of a hydrogen bond between the side-chain CO group of Gln15 and the main-chain NH group of Trp94 (Table 2) and an increase in the hydrophobic area buried inside the protein (33 Å^2). The mutation Thr16 to Arg has been previously analysed in detail (Serrano *et al.*, 1990); the increase in stability upon mutation is due to better van der Waals' contacts with Tyr17 (0·2 kcal mol^{-1}) and to a favourable electrostatic interaction with Asp12. The His18 to Lys mutation has been described previously (Loewenthal *et al.*, 1992); the change in stability, which is pH-dependent, is due to a favourable interaction of the charged His residue with Trp94, and is less than 1 kcal mol^{-1} at pH 7·0 (Sali, 1990). Lys19 is located on the loop connecting the first and second α-helices of barnase. It is highly exposed to the solvent in barnase (Table 1) and makes an electrostatic interaction with Asp22. Arg19 in binase makes a hydrogen bond with the OH of Tyr13 (Table 2). There are two mutations in the second α-helix of barnase. The side-chain of Glu29 in barnase makes a hydrogen bond with the main-chain NH group of Ser28 (Table 2) that is not made by Gln29 in binase. Gln31 is highly exposed in barnase and the equivalent residue in binase (Ser31) makes a hydrogen bond with the main-chain CO group of Ser28. In the small one-turn α-helix of barnase, there is one mutation, Asp44 to Glu. The side-chain of Asp44 is ill-defined in the crystal structure of barnase as is that of Glu44 in binase. Ile55 in the first β-strand is highly exposed to the solvent (Table 1), and the equivalent Val residue in binase makes similar van der Waals' contacts (Serrano *et al.*, 1992). Lys62, Gly65 and Lys66 are located on the guanosine-binding loop (Sevcik *et al.*, 1990). The side-chain of Lys62 makes a hydrogen bond with the side-chain CO group of Gln104. In binase, the side-chain of Arg62 makes a hydrogen bond with the main-chain CO of Ala104. Gly65 and Lys66 are highly exposed in barnase as well as in binase (Table 1). Thr79 is located in the loop connecting the second and third β-strands of barnase. Its side-chain does not make a hydrogen bond and it is highly exposed to the solvent, as is Val79 in binase (Table 1). There are three mutations in the third β-strand of barnase: Ser85 → Ala, Ile88 → Leu and Leu89 → Val. Ser85 is the first residue of the third β-strand of barnase and it is highly exposed to the solvent, as is Ala85 in binase (Table 1). The side-chain of Ile88 is completely buried and is the central residue of the main hydrophobic core of barnase (Serrano *et al.*, 1992), as is Leu88 in binase (Table 1). Leu89 is the central residue of the active-site hydrophobic core of barnase (Serrano *et al.*, 1992) and is completely buried as is Val89 in binase (Table 1). Gln104 is located in the loop between the fourth and fifth β-strands in barnase. The side-chain $O^{\varepsilon 1}$ of Gln104 makes a hydrogen bond with the side-chain N^{ζ} of Lys62 in barnase and is highly exposed (Table 1). The equivalent residue Ala104 in binase is also exposed to the solvent. Lys108 is in the C-terminal region of the protein. The side-chain of Lys108 in barnase makes a hydrogen bond with the CO main-chain group of Arg110, as does the side-chain $N^{\eta 1}$ group of Arg108 in binase.

(b) *Thermodynamic analysis*

The thermodynamic analysis, by urea denaturation, of the 17 mutants is shown in Table 3. With only one exception (His18 → Lys), the effect on the free energy of denaturation at pH 6·3 of all the single mutations is less than ±1 kcal mol^{-1}. Some of the mutations that increase the stability of the protein were selected, a triple and a multiple mutant were made and their effect on protein stability was determined (Table 3). The stability of binase was also determined (Table 3).

(c) *Activity*

It has been noted that there is an inverse correlation between stability and activity for mutations of some residues in the active site of barnase (Meiering *et al.*, 1992). There are no significant correlations found here for residues that are not involved in binding and catalysis (Table 4). However, we found that binase is 25-fold more active than barnase with respect to the substrate GpUp (Table 4). We also found that binase is fivefold less active than barnase with respect to the substrate RNA type XI from bakers' yeast (Table 4). The rates observed with RNA as substrate were identical at 1·5, 2 and 3 mg/ml RNA, indicating that V_{max} is measured at these concentrations. We find that rates vary with different batches of RNA substrate and comparisons between enzymes can be made only if the same batch of RNA is used for all measurements, as was done here.

4. Discussion

(a) *Analysis of changes in stability on mutation*

There is no specific localization of the differences between barnase and binase to any particular region of the protein. The 17 amino acid substitutions are

Table 3

Changes in the free energies of unfolding of wild-type barnase and the different mutants at the two regular α-helices of barnase

Mutant	m[a] (kcal mol^{-2})	$\Delta G_u^{H_2O}$[b] (kcal mol^{-1})	$[Urea]_{50\%}$[c] [m]	$\Delta\Delta G_u^{[urea]_{50\%}}$[d] (kcal mol^{-1})
Q15I	2·02	10·2	5·06	−0·96
T16R	1·98	9·6	4·84	−0·53
H18K	2·00	7·9	3·96	1·19
K19R	2·08	9·7	4·68	−0·21
E29Q	1·88	8·6	4·56	0·01
Q31S	2·05	9·1	4·44	0·25
D44E	1·84	8·5	4·61	−0·08
I55V	1·93	8·5	4·42	0·29
K62R	2·04	8·8	4·32	0·48
G65S	2·03	9·8	4·83	−0·51
K66A	2·09	9·8	4·70	−0·25
T79V	1·91	9·0	4·72	−0·29
S85A	2·02	9·1	4·51	0·12
I88L	1·98	8·8	4·42	0·28
L89V	1·92	8·5	4·43	0·27
Q104A	1·98	8·8	4·46	0·21
K108R	1·91	9·6	5·05	−0·93
Q15I/T16R/K19R	2·00	10·8	5·42	−1·66
Multiple[e]	1·86	11·8	6·34	−3·44
Binase[f]	2·02	10·2	5·04	−0·93
Barnase	1·92	8·8	4·58	0·00

The free energies of unfolding of wild-type protein and mutants were analysed by urea denaturation monitored by fluorescence.

[a] m is the slope of the linear denaturation plot, $-d\Delta G_u/d[urea]$.

[b] ΔG^{H_2O} was determined by urea denaturation of the proteins and extrapolation of the data to zero (denaturant).

[c] $[Urea]_{50\%}$ is the concentration of urea at which 50% of the protein is unfolded.

[d] $\Delta\Delta G_u^{[urea]_{50\%}}$ was determined by subtracting $^{[Urea]_{50\%}}$ for each mutant from that for wild type, and multiplying the difference by the average slope $\langle m \rangle$ using sloping baseline fitting ($\langle m \rangle = 1{\cdot}95$) (Horovitz *et al.*, 1992). Note that the values of $\Delta\Delta G_u^{[urea]_{50\%}}$ are fairly precise measurements, whereas the values of $\Delta G_u^{H_2O}$ are relatively imprecise due to a long extrapolation of data (see Kellis *et al.*, 1989; Serrano *et al.*, 1992, for a discussion of accuracy).

[e] The multiple mutant has the following mutations: Q15I, T16R, K19R, G65S, K66A and K108R.

[f] Binase was wild type and was a generous gift from Professor M. Ya. Karpeisky.

scattered throughout the protein; six of them are in the α-helices, four in the β-strands and six in loops and turns. Two of the differences occur at the central positions of the two hydrophobic cores of the protein, four of the differences are close to the residues involved in the binding of the guanosine base in the guanosine binding loop, and one is close to the catalytic His102 residue (Sevcik *et al.*, 1990). Seven of the mutations are not conservative: Gln15 → Ile, Thr16 → Arg, Gln31 → Ser, Gly65 → Ser, Lys66 → Ala, Thr79 → Val and Gln104 → Ala, and there are three Lys to Arg mutations (19, 62 and 108). All except three of the mutations (Gln15 → Ile, His18 → Lys and

Table 4

Activity of the mutants

Protein	V_m (RNA)[a] ($\Delta A_{298\cdot5}$ s^{-1} M^{-1})	K_m(GpUp)[b] (μM)	k_{cat}(GpUp)[b] (s^{-1})	k_{cat}/K_m(GpUp)[b] (s^{-1} M^{-1})
Barnase	$(1{\cdot}50 \pm 0{\cdot}10) \times 10^5$	21·7 ± 1·2	58·5 ± 1·5	$(2{\cdot}67 \pm 0{\cdot}28) \times 10^6$
Q15I/T16R/K19R	—	19·0 ± 0·2	54·9 ± 0·3	$(2{\cdot}90 \pm 0{\cdot}06) \times 10^6$
Multiple[c]	—	26·5 ± 1·9	69·9 ± 1·5	$(2{\cdot}64 \pm 0{\cdot}20) \times 10^6$
W-T binase[d]	—	5·5 ± 1·3	403 ± 45	(73 ± 19) $\times 10^6$
Synthetic binase[d]	$(2{\cdot}78 \pm 0{\cdot}06) \times 10^4$	6·8 ± 0·8	437 ± 11	$(64{\cdot}3 \pm 7{\cdot}7) \times 10^6$

All experiments were performed at 25°C.

[a] Activity with bakers' yeast RNA, type XI, was determined in 100 mM Tris·HCl (pH 8·5) containing 100 μg/ml RNase-free BSA.

[b] Activity with GpUp was determined in 100 mM sodium acetate (pH 5·8), containing 100 μg/ml RNase-free BSA.

[c] The multiple mutant has the following mutations: Q15I, T16R, K19R, G65S, K66A and K108R.

[d] Synthetic binase has a Gln residue at position 2 that is not present in wild-type binase. The concentration of protein was determined spectrophotometrically at 280 nm based on the absorption coefficient for barnase, where $\varepsilon_{0\cdot1\%} = 2{\cdot}2$ (Loewenthal *et al.*, 1991). The kinetic analyses were performed as previously described (Day *et al.*, 1992; Mossakowska *et al.*, 1989).

Table 5
Additivity of changes in stabilization energies of the mutants

Protein	$\Delta\Delta G_{wt\ barnase}$ (kcal mol^{-1})[a]	$\Sigma\Delta\Delta G_{wt}$ (kcal·mol^{-1})[b]
Binase	−0·9	−0·7
Q15I/T16R/K19R	−1·7	−1·7
Multiple[c]	−3·4	−3·4

[a] $\Delta\Delta G_{wt\ barnase}$, the change in stabilization energy, was calculated as indicated for Table 1.
[b] $\Sigma\Delta\Delta G_{wt}$ was calculated by summing the effects of changes in stability of barnase of the single mutations.
[c] The multiple mutant has the following mutations: Q15I; T16R; K19R, G65S, K66A and K108R.

Lys108 → Arg) have an effect on protein stability that ranges from −0·5 to 0·5 kcal mol^{-1}.

(i) *The mutations that hardly affect the stability of barnase*

These are Glu29 → Gln, Asp44 → Glu and Ser85 → Ala. The three are conservative mutations of solvent-exposed residues. The mutation of Glu29 to Gln results in a decrease in stability because of breaking a hydrogen bond with the NH main-chain group of residue Thr26 and an increase in stability because of the elimination of an electrostatic repulsion between the side-chains of Asp54 and Glu29 (Sancho *et al.*, 1992) and an increase in the entropic freedom of the side-chain of Gln29 compared with that of Glu29. The destabilizing effect is also partly counterbalanced by the increase in solvent exposure of the main-chain NH group of Thr26 (from 4 to 29%). Mutation of Asp44 to Glu does not result in any significant change in the interactions of the side-chain of Asp44 with the rest of the molecule since, in both cases, the side-chains are highly mobile and do not interact with any residue in particular. Finally, the Ser85 → Ala mutation has a destabilizing effect due to a decrease in the hydrophobic surface (6 Å^2) and a stabilizing effect because the OH side-chain group of Ser85 is partly buried and screened from solvent without being hydrogen bonded within the protein (solvent exposure = 18 Å^2).

(ii) *The mutations that increase the stability of barnase*

These are Gln15 → Ile, Thr16 → Arg, Lys19 → Arg, Gly65 → Ser, Lys66 → Ala, Thr79 → Val and Lys108 → Arg. It is interesting that none of these changes takes place in the β-strands. Five of the changes occur in loops and in all the cases solvent-exposed residues are involved. Two of the stabilizing mutations increase the hydrophobic surface buried (Gln15 → Ile and Thr79 → Val). Mutation of Gln15 to Ile breaks one internal hydrogen bond, leaving a hydrogen bond donor group without acceptor. This could result in a decrease of protein stability about 1·5 kcal mol^{-1} (Fersht, 1987). The concomitant increase in the hydrophobic surface buried of 33 Å^2 should result in a net increase in the stabilization of the protein (Matsumura *et al.*, 1988). Mutation of Thr79 to Val increases the hydrophobic surface buried by around 3 Å^2, and there is no effect due to the mutation of the side-chain OH group to a methylene group, since the OH is almost 100% exposed to the solvent. The mutation Gly65 → Ser decreases the entropy of the unfolded state, and the stabilization found is in the range previously predicted for this type of mutation (Matthews *et al.*, 1987). One of the mutations, Thr16 → Arg, introduces a favourable electrostatic interaction with Asp12 and makes better van der Waals' contacts with Tyr17 (Serrano *et al.*, 1990). Three of the mutations (Lys19 → Arg, Lys108 → Arg and Lys66 → Ala) are of the type predicted, from comparing thermo-stable and normal proteins, to decrease flexibility and increase thermo-stability (Menendez-Arias & Argos, 1989).

(iii) *The mutations that decrease the stability of barnase*

These are His18 → Lys, Gln31 → Ser, Ile55 → Val, Ile88 → Leu, Leu89 → Val and Gln104 → Ala. The decrease in stability on mutation of His18 → Lys results from losing a favourable interaction of the charged ring of the His residue with Trp94 (Loewenthal *et al.*, 1992). Mutation of Gln31 to Ser results in the formation of a hydrogen bond between the side-chain OH group of Ser31 and the main-chain CO of Ser28. There is a destabilizing effect from a decrease in the hydrophobic surface buried (17 Å^2) and a stabilizing effect from the NH_2 side-chain group of Gln31 being partly buried without being hydrogen bonded to the protein (solvent-accessible area of 37 Å^2). Mutation of Ile55 to Val decreases the hydrophobic surface buried by 4·6 Å^2. Mutation of Ile88 to Leu maintains the hydrophobic surface buried but changes the packing arrangement slightly. Mutation of Leu89 to Val decreases the hydrophobic surface buried and changes the packing arrangement of the surrounding residues.

(b) *Co-operativity of the effects of mutation*

The folding of a protein is highly co-operative. The effect on protein stability of many of the interactions between two or more residues may also be co-operative, and so breaking a particular interaction may weaken a further set of interactions (Horovitz *et al.*, 1991). There seems to be no co-operativity connected with the 17 substitutions between barnase and binase, since the sum of the effects of the single mutations on the stability of the protein is very close to the value obtained for the difference in stability between the two proteins. Moreover, the stability of a triple or sextuple mutant is similar to the sum of the effects of the single mutations (Table 4). This is particularly interesting in the case of mutations of residues that are close to each other and in the same element of secondary structure (Gln15 → Ile and Thr16 → Arg in the first α-helix; Glu29 → Gln and Gln31 → Ser in the second α-helix; Gly65 → Ser and Lys66 → Ala in

the guanosine binding loop and Ser85 → Ala, Ile88 → Leu and Leu89 → Val in the third β-strand). Co-operativity is, perhaps, expected because of stabilisation or destabilisation of secondary structure elements. The effect of the mutations located in the same secondary structure element has, in general, the same algebraic sign and, at least for the first α-helix and guanosine binding loop, they are additive. This suggests that the stabilizing or destabilizing effect of a single mutation in a secondary structural element does not affect the strength of the interactions made with the rest of the protein by another residue in the same secondary structural element.

In a similar study on subtilisin BPN′, using a different approach, it was found that small independent changes in the stability of the protein were additive and resulted from very subtle changes in the structure of the protein. As in barnase to binase, the increase in stability is due to several different effects, ranging from better van der Waals' contacts to improved hydrogen bonding (Pantoliano *et al.*, 1989). Also there are two close substitutions (positions 217 and 218) that do not exhibit co-operativity. Not all of the interactions found in a protein are co-operative, and probably one of the more important lines of research on protein stability in the future will be the elucidation of which interactions are co-operative and which are not.

(c) *Protein activity*

It is suggested that at mesophilic temperatures thermo-stable proteins are less flexible (Vihinen, 1987). Flexibility is essential for the regulation and function of several proteins. Consequently, thermostable proteins should have an optimum activity at higher temperatures than for the equivalent mesophilic proteins. A protein engineering study on barnase indicates that there is an inverse correlation between stability and activity for some of the interactions that take place in the active site (Meiering *et al.*, 1992). The analysis of the activity of our more stable multiple mutants indicates that there need not be an inverse relationship between activity and stability for mutations of residues that are not in the active site and thus it is possible to have more stable proteins with the same activity at the same temperature as wild-type protein.

The one odd result is that the activity of binase towards the dinucleotide, GpUp, is 25 times higher than that of barnase (at pH 5·8) and the stable multiple mutants, and that the activity of binase towards RNA is some five times lower than that of barnase (at pH 8·5). This is true for both wild-type binase and binase expressed from an artificially constructed gene that does not have the deletion, Gln2 → Δ, found in wild-type. There are five differences between barnase and binase that are located close or in the guanosine binding loop (Ile55 → Val, Lys62 → Arg, Gly65 → Ser, Lys66 → Ala and Leu89 → Val; Sevcik *et al.*, 1990), which could account for this difference, either independently or co-operatively. The pH dependence of the activity of barnase towards dinucleotides is sharply bell-shaped (Mossakowska *et al.*, 1989). A complete analysis of the pH dependence of activities of mutants will be required for more detailed studies. This, and the construction of further hybrids, is the subject of ongoing investigation in this laboratory.

(d) *Protein evolution*

One of the central questions in protein evolution is how the changes in the amino acid sequence of a protein result in changes in stability and structure and, therefore, in its function. It is generally assumed that small changes in the sequence of a protein should not have large effects on its structure or stability and that changes on the surface of a protein should have a smaller impact than those in buried regions. Consequently, and according to the neutral theory of evolution, the great majority of mutant substitutions will be caused by random fixation through sampling drift of selectively neutral mutants (Kimura, 1991). It follows from this theory, that unless there is a selective pressure for changing the stability of a protein, the stability of two related proteins should be similar and the effect of single or multiple mutations along the evolutionary pathway should not be bigger than the difference in stability between the two proteins (neutral corridor) (Malcom *et al.*, 1990). Under selective pressure to increase the stability of an enzyme, there will be an accumulation of small stabilizing mutations that will result in an overall increase of the stability of the protein. This is in agreement with the analysis of thermo-stable enzymes that has indicated that many small changes over the entire polypeptide chain are responsible for protein thermo-stabilization (Argos *et al.*, 1979).

Our results do not prove that the evolution from barnase to binase, or *vice versa*, has followed a neutral corridor, since we do not have other proteins intermediate between the two. However, the observation that the effect on protein stability of every one of the mutants is not higher or lower than the difference in stability between barnase or binase, and that the effects are additive, could be interpreted as there being an evolutionary pressure to maintain the stability of these proteins within a narrow range of ±10% of their total stability. These results would then be in good agreement with the neutral theory of evolution. Alternatively, there could be simply selective pressure to maintain stability above a certain threshold level. There will then be no further pressure to increase stability or to retain any mutations that do increase stability. Our results do, however, show that it is possible to increase significantly the stability of a protein by the addition of stabilizing mutations.

5. Conclusions

This study suggests a method to improve the stability of proteins without any prior knowledge

about their structure or the principles that govern protein stability. Choose two highly related proteins, make all the individual mutants between the two, determine the changes in stability, select the mutations that increase stability and then construct the multiple mutants that contain the favourable mutations. This is most likely to work with proteins that have not diverged too much, so minimizing the number of mutations to be made and the likelihood of many co-operative changes in structure or stability, as has been found in the case of highly divergent proteins (< 45% homology; Imanaka *et al.*, 1986).

L.S. was supported by an EC fellowship.

References

Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G. & Tratschin, J. D. (1979). Thermal stability and protein structure. *Biochemistry*, **18**, 5698–5703.

Baudet, S. & Janin, J. (1991). Crystal structure of a barnase d(GpC) complex at 1·9 Å resolution. *J. Mol. Biol.* **214**, 123–132.

Bycroft, M., Ludvigsen, S., Fersht, A. R. & Poulsen, F. M. (1991). Determination of the three dimensional solution structure of barnase using nuclear magnetic resonance spectroscopy. *Biochemistry*, **30**, 8697–8701.

Clackson, T., Güssow, D. & Jones, P. T. (1991). *PCR: A Practical Approach* (McPherson, M. J., Quirke, P. & Taylor, G. R., eds), pp. 187–214, IRL Press, Oxford.

Day, A. G., Parsonage, D., Ebel, S., Brown, T. & Fersht, A. R. (1992). Barnase has subsites that give rise to large rate enhancements. *Biochemistry*, **31**, 6390–6395

Fersht, A. R. (1987). The hydrogen bond in molecular recognition. *Trends Biochem. Sci.* **12**, 301–304.

Fersht, A. R. & Serrano, L. (1993). Principles of protein stability determined from protein engineering experiments. *Curr. Opin. Struct. Biol.* **3**, 75–83.

Hartley, R. W. (1975). A two state conformational transition of the extracellular ribonuclease of *Bacillus amyloliquefaciens* (barnase) induced by sodium dodecyl sulfate. *Biochemistry*, **14**, 2367–2370.

Hill, C., Dodson, G., Heinemann, U., Saenger, W., Mitsui, Y., Nakamura, K., Borisov, S., Tischenko, G., Polyakov, K. & Pavlovsky, S. (1983). The structural and sequence homology of a family of microbial ribonucleases. *Trends Biochem. Sci.* **8**, 364–369.

Horovitz, A., Serrano, L., Avron, A., Bycroft, M. & Fersht, A. R. (1991). Strength and co-operativity of contributions of surface salt-bridges to protein stability. *J. Mol. Biol.* **216**, 1031–1044.

Horovitz, A., Matthews, J. & Fersht, A. R. (1992). α-Helix stability in proteins. I. Factors that influence stability at internal positions. *J. Mol. Biol.* **227**, 560–568.

Imanaka, T., Shibazaki, M. & Takagi, M. (1986). A new way of enhancing the thermostability of proteases. *Nature (London)*, **324**, 695–697.

Kellis, J. T., Jr, Nyberg, K., Sali, D. & Fersht, A. R. (1988). Contribution of hydrophobic interactions to protein stability. *Nature (London)*, **333**, 784–786.

Kellis, J. T., Jr, Nyberg, K. & Fersht, A. R. (1989) Energetics of complementary side-chain packing in a protein hydrophobic core. *Biochemistry*, **28**, 4914–4922.

Kimura, M. (1991). Recent development of the neutral theory viewed from the Wrightian tradition of theoretical population genetics. *Proc. Nat. Acad. Sci., U.S.A.* **88**, 5969–5973.

Loewenthal, R., Sancho, J. & Fersht, A. R. (1991). Fluorescence spectrum of barnase: Contributions of three tryptophan residues and a histidine-related pH-dependence. *Biochemistry*, **30**, 6775–6779.

Loewenthal, R., Sancho, J. & Fersht, A. R. (1992). Histidine–aromatic interactions in barnase: Elevation of histidine pK_a and contribution to protein stability. *J. Mol. Biol.* **224**, 759–770.

Malcom, B. A., Wilson, K. P., Matthews, B. W., Kirsch, J. F. & Wilson, A. C. (1990). Ancestral lysozymes reconstructed, neutrality tested, and thermostability linked to hydrocarbon packing. *Nature (London)*, **345**, 86–89.

Matsumura, M., Becktel, W. J. & Matthews, B. W. (1988). Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile3. *Nature (London)*, **334**, 406–410.

Matthews, B. W., Nicholson, H. & Becktel, W. J. (1987). Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Nat. Acad. Sci., U.S.A.* **84**, 6663–6667.

Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C. & Jack, A. (1982). Molecular structure of a new family of ribonucleases. *Nature (London)*, **297**, 162–164.

Meiering, E. M., Serrano, L. & Fersht, A. R. (1992). Effect of active site residues in barnase on activity and stability. *J. Mol. Biol.* **225**, 585–589.

Menendez-Arias, L. & Argos, P. (1989). Engineering protein thermal stability: Sequence statistics point to residue substitutions in alpha-helices. *J. Mol. Biol.* **206**, 397–406.

Miller, S., Janin, J., Lesk, A. M. & Chothia, C. (1987). Interior and surface of monomeric proteins. *J. Mol. Biol.* **196**, 641–656.

Mossakowska, D. E., Nyberg, K. & Fersht, A. R. (1989). Kinetic characterization of the recombinant ribonuclease from *Bacillus amyloliquefaciens* (barnase) and investigation of key residues in catalysis by site-directed mutagenesis. *Biochemistry*, **28**, 3843–3850.

Mrabet, M. N., Vandenbroeck, A., Vandenbrande, I., Stanssens, P., Laroche, Y., Lambeir, A. M., Matthijssens, G., Jenkins, J., Chiadmi, M. & Vantilbeurgh, H. (1992). Arginine residues as stabilising elements in proteins. *Biochemistry*, **31**, 2239–2253.

Paddon, C. J. & Hartley, R. W. (1987). Expression of *Bacillus amyloliquefaciens* extracellular ribonuclease (barnase) in *Escherichia coli* following an inactivating mutation. *Gene*, **53**, 11–19.

Pantoliano, M. W., Whitlow, M., Wood, J. F., Dodd, W., Hardman, K. D., Rollence, M. L. & Bryan, P. N. (1989). Large increases in general stability of subtilisin BPN′ through incremental changes in the free energy of unfolding. *Biochemistry*, **28**, 7205–7213.

Pavlovsky, A. G., Vagin, A. A., Vainstein, B. K., Chepurnova, N. K. & Karpeisky, M. Ya. (1983). Three-dimensional structure of ribonuclease from *B. intermedius* 79 at 3·2 Å resolution. *FEBS Letters*, **162**, 167–170.

Pavlovsky, A. G., Sanishvili, R. G., Strkopytov, B. V., Vainshtein, B. K., Vagin, A. A., Chepurnova, N. K. & Borisova, S. N. (1989). The structure of 2 crystal modifications of *Bacillus intermedius* ribonuclease. *Kristallografiya*, **34**, 137–142.

Sali, D. (1990). Interactions contributing to barnase stability. Ph D thesis, Cambridge University.

Sancho, J., Serrano, L. & Fersht, A. R. (1992). Histidine residues at the N-termini of α-helices: perturbed pK_as and protein stability. *Biochemistry*, **31**, 2253–2258.

Sayers, J. R., Schmidt, W. & Eckstein, F. (1988). 5′–3′ Exonucleases in phosphorothioate-based oligonucleotide directed mutagenesis. *Nucl. Acids Res.* **16**, 791–802.

Serrano, L. & Fersht, A. R. (1989). Capping and α-helix stability. *Nature (London)*, **342**, 296–299.

Serrano, L., Horovitz, A., Avron, B., Bycroft, M. & Fersht, A. R. (1990). Estimating the contribution of engineered surface electrostatic interactions to protein stability using double-mutant cycles. *Biochemistry*, **29**, 9343–9352.

Serrano, L., Kellis, J. T., Cann, P., Matouschek, A. & Fersht, A. R. (1992). The folding of an enzyme. II. Substructure of barnase and the contribution of different interactions to protein stability. *J. Mol. Biol.* **224**, 783–804.

Sevcik, J., Sanishvili, R. G., Pavlovsky, A. G. & Polyakov, K. M. (1990). Comparison of active sites of some microbial ribonucleases: structural basis for guanylic specificity. *Trends Biochem. Sci.* **15**, 158–162.

Vihinen, M. (1987). Relationship of protein flexibility to thermostability. *Protein Eng.* **1**, 477–480.

Vriend, G. (1990). WHAT IF: A molecular modeling and drug design program. *J. Mol. Graph.* **8**, 52–56.

Edited by J. Karn