Engineering enzymes for stability

Andrew Shaw and Richard Bott

There have been many recent developments in elaborating the approaches for stabilizing enzymes by stabilizing the folding state, destabilizing the unfolded state and altering the kinetics of unfolding. However, these represent a series of rules of thumb rather than the reliable principles that would be expected of 'engineering'. Stability is taken to include thermodynamic stability as measured by reversible denaturation, and kinetic stability as measured by the unfolding rate for enzymes that are subject to irreversible denaturation. As such, the factors that affect stability of the folded state versus the unfolded state, as well as factors affecting rates of folding and unfolding, all play a role in maintaining stability. Recent studies on the role of kinetics and the effect that site-specific substitutions have on transition-state free energies add to our understanding of the factors that determine whether or not a particular substitution will result in the measurable stabilization of a protein.

Address
Genencor International, 925 Page Mill Road, Palo Alto, CA 94304, USA

Current Opinion in Structural Biology 1996, 6:546–550

Abbreviations
CI2 chymotrypsin inhibitor 2
PLA2 phospholipase A2

Introduction

Enzymes are catalysts that can enhance reaction rates by many orders of magnitude, usually display exquisite specificity and have, therefore, many potential biomedical, chemical and industrial applications. For these applications, enzymes are frequently required to catalyze reactions in distinctly different environments to those in which they have evolved to function in nature. Thus, there is an important need to engineer enzymes that can function in non-natural environments, so that their full potential can be realized. A major limitation to enzyme function in non-natural environments is stability. The enhancement of enzyme stability is therefore an important goal of protein engineering.

In many industrial applications, stability is defined as having a sufficient lifetime under specified conditions to complete a reaction. As such, the factors affecting kinetic stability are as important as those that affect the thermodynamic equilibrium. Simple model systems, such as phospholipase A2 [1•], chymotrypsin inhibitor 2 [2•], GCN4 [3•], and P22 arc repressor [4•] have a reversible two-state folding mechanism that is amenable to rigorous thermodynamic analysis, and have been used to understand how the folded state of a protein can be stabilized. This work complements the very extensive work carried out on more complex systems, for example, T4 lysozyme [5], staphylococcal nuclease [6••] and subtilisin [7,8]. Sequence and structural information on enzymes from extremophiles, which are often immensely stable, is becoming available, and provides further insights into the basis of enzyme stability [9•,10•]. There are now several recent examples that illustrate the role kinetics can play in stabilizing proteins [11–13]. From such information, engineering guidelines for stabilizing the folded state can be separated into thermodynamic factors (both entropic and enthalpic factors), for systems in rapid equilibrium, and kinetic factors that alter the stability of transition states in the folding/unfolding pathway, for systems that slowly equilibrate or irreversibly denature. This review includes several recent papers that have successfully applied a number of stabilization strategies, along with recent results looking at the effects of site-specific substitutions on folding kinetics and the analysis of thermostability found among evolutionarily related enzymes. These involve several common themes relating to stabilization that alters stability by altering the relative thermodynamic energies of the folded and unfolded states, 'equilibrium stability' and stabilization as measured by increased lifetime that is a result of altering unfolding kinetics or 'kinetic stability'.

Entropic stabilization factors

Entropy is a driving force in protein folding. When unfolded, the polypeptide has more degrees of freedom, but is extensively hydrated, particularly at hydrophobic residues around which water clathrate structures form, so that the entropy of the solvent is lowered. When folded, the protein has much fewer degrees of freedom, but sequestration of hydrophobic groups releases bound water molecules, so that the entropy of the solvent is increased. The result is a net increase in the entropy of the system in the folded state. Makhatadze and Privalov [14] have presented evidence that the hydration of polar groups also makes major contributions to entropy changes. Mutations that decrease the entropy of the system of the unfolded protein, or increase the entropy of the system of the folded protein, will result in a larger ΔS for folding, which, in the absence of complementary enthalpic contributions, will stabilize the structure.

A strategy for lowering the entropy of the unfolded protein is to reduce the degrees of freedom in the main chain. One can replace glycines with residues that have side chains, such as alanine, and replace residues in turns with proline, both of which reduce the conformational degrees of freedom. This approach has been successful in T4 lysozyme [5], and *Bacillus cereus* oligo-1,6-glucosidase [15]. Corroboration for the rationale of this approach can be seen

from protease substrate interaction studies. We assume that the thermodynamic factors that affect the stability of a polypeptide substrate binding to a protease are the same as those that hold different segments of a protein together. In a recent study on the binding of various peptide-like inhibitors with thrombin, it was shown that inhibitors that have side chains with more degrees of freedom in solution bind less tightly than those that have more restricted side chains [16••]. The lower affinity has been attributed to the higher entropy of inhibitors with side chains with more degrees of freedom when unbound relative to those having more restricted side chains in a manner analogous to the factors affecting the free energy of the unfolded state in proteins.

Thermostable enzymes, such as the thermostable triosephosphate isomerase from *Bacillus stearothermophilus* [17], have fewer internal cavities than their mesophilic counterparts, so that the removal of water trapped in internal cavities increases the entropy of the folded structure. Also, filling in cavities with larger residues will result in an unfolded polypeptide with a larger side chain to hydrate, lowering its entropy. Strategies devised to fill in internal cavities have been successful for subtilisin [18,19]. In addition, thermostable enzymes often have shorter loops, leading to folded molecules that are more compact than their mesophilic counterparts [20•] and often have larger, more hydrophobic interfaces [17]. This results in a smaller overall surface to be hydrated, leading to an increase in the entropy of the folded protein. Disulfide bonds are believed to play an important role in protein stabilization by increasing the entropy of folding by decreasing the entropy of the unfolded state. This was achieved in T4 lysozyme by Perry and Wetzel [21] in perhaps the first example of engineered, stabilizing site-specific substitutions.

Enthalpic stabilization factors

The free energy of a folded protein is lowered by favorable enthalpic interactions within the structure. These include extensive hydrogen bonding [22], complementary van der Waals interactions [23] and electrostatic charge interactions, such as ion pairs [24]. Homology modeling predicted that d-glyceraldehyde-3-phosphate from *Thermotoga maritima* has 12 more surface ion pairs than the equivalent enzyme from *B. stearothermophilus* [25]. The interaction of barnase with the inhibitor barstar forms a very tight complex ($K_D = 10^{-14}$ M) that may also serve as a paradigm for internal protein stabilization. It was found from extensive mutagenesis studies that the interaction between barnase and barstar is stabilized predominantly by a series of coupled ion-pair interactions [26].

Helices figure prominently in the folding intermediates of the prosubtilisin–subtilisin complex [27••], chymotrypsin inhibitor 2 (CI2) [2•], and α-lactalbumin [28] and are factors in thermodynamic stabilization and folding kinetics. Certain amino acids appear with higher frequency in different elements of secondary structures such as the N caps and C caps of α helices [29]. The introduction of a negatively charged side chain at the N cap, which can neutralize the partial dipole created by the unpaired amide protons, has been shown to increase stability in T4 lysozyme [30]. Similar results have been reported for the complementary removal of negatively charges side chains near the C cap of α helices in ribonuclease T1 [10•]. Comparisons of the amino acid preferences of helices from thermophiles with those from mesophiles have been made [9•,20•]. Tyrosine, glycine and glutamine have increased frequency in the α helices of thermophilic proteins whereas valine, glutamic acid, histidine, cyteine and aspartic acid have decreased frequency. The increased frequency of glycine residues at the C termini of seven helices in thermophilic citrate synthetases was attributed to the capacity of glycine to adopt the α_L conformation at the C cap without strain [20•]. A related analysis of the N- and C-capping preferences for all 20 amino acids in α-helical peptides [31] found that the N cap preferences of peptides and protein are retained, but that glycine was not preferred in the C cap of peptide helices. This may reflect a greater need for correct helix termination in proteins and its role in structure stabilization.

Practical experience

The potential for stabilization can be seen in three recent reports in which stabilizing substitutions have been combined in T4 lysozyme [32••], hen lysozyme [33••] and subtilisin BPN′ [34••]. In T4 lysozyme, seven substitutions were made: three to stabilize the partial positive charge at the N caps of α helices (Ser38Asp, Asn116Asp and Asn144Asp); two to increase the entropy of the unfolded state (Ala82Prc and Ala41Val); one on the basis of helix propensity (Val131Ala); and one to optimize hydrophobic interaction (Ile3Leu). They were additive and increased the melting temperature (T_m) by 8.3°C. In hen lysozyme, six substitutions were combined to increase the T_m by 10.5°C: three improved side chain packing (Ala31Val, Ile55Leu and Ser91Thr); one replaced a negatively charged residue in the C cap of an α helix (Asp101Ser); and two (His15Leu and Arg114His) were selected by homology from the more thermostable pheasant lysozyme. In subtilisin BPN′ six substitutions were introduced to stabilize a variant in which the strong calcium-binding site had been removed: two were intended to introduce a disulfide bond tethering the N-terminal segment (Ser3Cys and Gln206Cys); one replaced a proline with a serine (Pro5Ser), two adjusted charge interactions (Gln2Lys and Lys43Asn); and Ala73Lys increased hydrophobic interactions. These six substitutions increased the kinetic stability 1000-fold, as measured by half-life at stringent conditions. These studies combine substitutions utilizing both entropic and enthalpic stabilization principles and demonstrate that effects at individual sites that are widely dispersed in the three-dimensional structures of these enzymes can be combined to significantly improve stability.

Although success in stabilizing proteins can be impressive, the approaches described above are not always successful. In subtilisin, for example, several disulfide bridges were introduced with very little stabilization and sometimes even destabilization [35,36]. Each of the seven disulfides of phospholipase A2 (PLA2) have been removed [1•] with the following results: deletion of one of the seven, Cys84Ala–Cys96Ala, resulted in an unstable enzyme that could not fold correctly whereas deletion of one of the remaining six (Cys27Ala–Cys123Ala) increased the stability of the enzyme by 2.4 kcal mol^{-1}. These results and similar work suggesting that the disulfide bridge in ribonuclease T1 forms late or at the end of folding [37] underscores the lack of predictability of the effects of disulfide bonds.

The lack of predictability seen for disulfides is also found for other strategies, such as the introduction of salt bridges [38] and modifications to neutralize the dipole at the N caps of α helices in subtilisin (R Bott, unpublished data). It is possible that the successes represent instances where optimal side chain packing required for maximal enthalpic stabilization is achieved and that failures may reflect how exacting geometry that must be in order to observe stabilization. It has been suggested that the entropic cost of introducing large charged side chains to form salt bridges may offset the enthalpic benefits [39]. However, the kinetics of folding is another factor that needs to be considered for irreversible and slowly unfolding proteins.

Folding kinetics

Several simple systems have been identified [2•–4•] in which folding and unfolding is fully reversible and that have only one transition state. One of the most extensively studied is CI2, is a single polypeptide chain of 64 residues. Itzhaki *et al.* [2•] have measured relative changes in free energies, ΔG, of unfolding, and changes in kinetics of folding and unfolding, for mutants at 45 of the 64 residues. By analyzing the rate of folding and unfolding for native and variants, the authors determined whether the substitution altered the free energy of the transition state. With a few noteworthy exceptions, in the N-terminal part of the helix, the majority of substitutions did not alter the free energy of the folding transition state. Essentially similar results were obtained with GCN4 [3•] and with P22 arc repressor [4•]. From these results, it can be concluded that for a majority of the substitutions, in these two-state systems, the stabilization effects can be understood on the basis of alterations in the relative free energies of the folded and unfolded states and that the kinetics of folding are unchanged. However, the folded and unfolded state of many enzymes are not in dynamic equilibrium on a short timescale. Therefore, the stability is likely to be controlled by kinetics, and manipulation of the relative energies of folded and transition states are required.

Even in simple two-state systems, there are segments that are ordered in the transition state. In these cases, mutations affect the energy of the transition state, and thus may or may not alter the rate of unfolding, depending on whether the transition state resembles the folded state for those segments. This is important, because for many proteins, the folded and unfolded proteins are not in rapid equilibrium, for example, mature subtilisin. In these cases, stability will be determined in whole or in part by kinetics, that is, whether the rate of folding versus unfolding increases or decreases. Manipulation of unfolding and folding kinetics will become increasingly important to protein engineering, and these insights into the structures of folding transition states are very valuable. Larger, more complex proteins will often have folding intermediates and therefore a number of transition states, making kinetic stabilization more difficult to predict rationally. A good example is staphylococcal nuclease, in which both native and non-native folding intermediates have been detected [40••].

The structures of the transition state in the folding pathway of barnase have been well characterized [41,42]. It was possible to introduce disulfide-linking segments that form early and late in the folding pathway and this has provided clear evidence that the unfolding kinetics could be dramatically altered [11]. The disulfide-linking segments that break early in the folding pathway, for example, Ala43Cys–Ser80Cys, was shown to reduce unfolding by a factor of 170, whereas the disulfide-linking segments that break late, for example, Ser85Cys–His102Cys, after the rate-limiting step in unfolding reduces the unfolding rate by a factor of 17. Furthermore, the Ala43Cys–Ser80Cys variant that unfolds more slowly and was less stable than Ser85Cys–His102Cys. Two more examples are found with lysozyme [12] and glucoamylase [13]. In lysozyme, Asp–Gly sequences, which are chemically labile, were changed to Asp–Ala, to protect against thermal inactivation. Each of the changes decreased the thermodynamic stability of the enzyme. However, by suppressing the isomerization of the Asp–Gly sequence, the thermal inactivation rates at pH 4 and 100°C were lower for the mutant enzymes. Thus, under the conditions tested, the mutants were kinetically more stable than wild type.

Subtilisin is synthesized with a 77-residue prosegment and the hydrolysis of this prosegment to create a mature enzyme is believed to be the result of autolysis. The refolding of denatured mature subtilisin is extremely slow, with $t_{1/2}$ of refolding measured in weeks or longer [43]. In the presence of the prosegment, mature subtilisin readily renatures in less than a second [43]. The prosegment has therefore been termed a 'foldase'. Interestingly, a calcium binding site involving Gln2 as a ligand cannot be completely formed prior to hydrolysis. Calcium binding is postulated to serve as a trigger to actuate hydrolysis and stabilizes the structure after maturation. For proteins that

have a slow refolding rate, this work is of considerable importance.

Conclusions

There are numerous recent examples of site-specific mutants that increase the stability of proteins [1•–4•,11–13, 32••–34••], and, at least for simple systems, the rationale is clear. It has also been amply demonstrated that such mutants can be combined and the stability increases can be additive [32••–34••]. However, application of these general guidelines also result in numerous failures, most of which go unpublished. An important question, therefore, is why do the guidelines work only some of the time? These guidelines have been proposed on the basis of relatively simple systems, in which the folded and unfolded states are in dynamic equilibrium. If the ΔG of folding is more negative, and the free energy of the transition state is unaffected, then the activation energy for unfolding increases and the rate decreases [3•]. Variants having the above properties have been referred to as equilibrium folding mutants [44]. However, if the substitution lowers the free energy of the folded, unfolded or folding transition states simultaneously, it is not so straightforward. It has been suggested that the unfolded state is really an ensemble of compact structures in staphylococcal nuclease, and site-specific substitutions can alter the free energy of at least some of the transition states [6••]. Altering the energy of the folding transition state will alter the kinetics of folding [45]. Conversely, altering the energy of the unfolding transition states will alter kinetics of unfolding effecting kinetic stability. Therefore, the effects of substitutions not only on the folded and unfolded states but also on the transition states and intermediates need to be considered. When this information can be obtained, reliable prediction of substitutions that will result in altered stability will be possible.

Acknowledgements

We are very grateful to Anthony Day, James Kellis and Christopher Murray for critical reading of the manuscript and for bringing additional relevant references to our attention.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Zhu H, Dupureur CM, Zhang X, Tsai M-D: **Phospholipase A2 engineering. the roles of disulfide bonds in structure, conformational stability and catalytic function.** *Biochemistry* 1995, **34**:15307–15314.

Each of seven disulfide bridges in the 123 amino acid PLA2 have been replaced pairwise by the mutation of Cys→Ala cysala. NMR structural analysis of the six folded variants found that although the variants retained enzymatic activity, many were in less-ordered states than the native enzyme.

2. • Itzhaki LS, Otzen DE, Fersht AR: **The structure of the transition state for folding of chymotrypsin inhibitor 2 analysed by protein engineering methods: evidence for a nucleation–condensation mechanism for protein folding.** *J Mol Biol* 1995, **245**:260–288.

A very concise analysis of numerous variants of CI2 that have been analyzed for their relative conformational stability and alterations in their folding and unfolding kinetics. The focus of the paper is to identify residues that are already folded in the transition state and thus can be used to define the structure of the transition state. The finding is that residues in the N-terminal helix may already fold in the transition state. For the purposes of this review, the finding that most are not folded in the transition state is more relavant.

3. • Sosnick TS, Jackson S, Wilk RR, Englander SW, DeGrado WF: **The role of helix formation in the folding of a fully α-helical coiled coil.** *Proteins* 1996, **24**:427–432.

An analysis of the folding and dimerization of GCN4. The very concise discussion of the relevance of folding kinetics to the analysis of transition states is well worth reading.

4. • Milla ME, Brown BM, Waldburger CD, Sauer RT: **P22 arc repressor: transition state properties inferred from mutational effects on the rates of protein unfolding and refolding.** *Biochemistry* 1995, **34**:13914–13919.

Another analysis of folding kinetics and its relevance to stability. This study, taken in conjuction with [2•] and [3•] makes a compellling statement of the limited amount of ordered structure in the folding transition states of these simple folding systems.

5. Matthews BW: **Structural and genetic analysis of protein stability.** *Annu Rev Biochem* 1993, **62**:139–160.

6. •• Shortle D: **The denatured state (the other half of the folding equation) and its role in protein stability.** *FASEB J* 1996, **10**:27–34.

This is an excellent review, examining stability from the point of view of the relative stability of the unfolded state. Taken in conjuction with the references cited within, this review constitutes a must read for all who want to stabilize folded proteins.

7. Wells JA, Estell DA: **Subtilisin – an enzyme designed to be engineered.** *Trends Biochem Sci* 1988, **13**:291–297.

8. Bott R, Betzel C (Eds): New York: Plenum Press; 1996:in press. **Subtilisin symposium: practical protein engineering.**

9. • Warren GL, Petsko GA: **Composition analysis of α-helices in thermophilic organisms.** *Protein Eng* 1995, **8**:905–913.

The authors compared the statistical preference for particular amino acids in α-helices in 19 thermophilic organisms with the preferences of mesophilic enzymes. Their findings were also predicted on the basis of Δs, where s is the Zimm–Bragg helix propagation value. These results are taken in conjunction with those of Muir *et al.* [20] and should provide some insight into the stabilization of helices which figure prominantly in the folding pathways of a number of proteins.

10. • Walter S, Hubner B, Hahn U, Schmid FX: **Destabilization of a protein helix by electrostatic interactions.** *J Mol Biol* 1995, **252**:133–143.

The examination of the stabilization of the α-helix C cap by removal of negatively charged residues. While these results are useful for a generic understanding of stabilization at neutral pH however, the substitutions did not affect the apparent stability of the enzyme under native conditions because Ribonuclease T1 is active *in vivo* under acidic conditions where the substitution of acidic groups such as aspartic acid are not charged. Replacement of glutamic and aspartic acid residues with glutamine and asparagine, individually and additively, stabilized the enzyme above pH 5.

11. Clarke J, Fersht AR: **Engineering disulfide bonds as probes of the folding pathway of barnase: increasing stability of protein against the rate of denaturation.** *Biochemistry* 1993, **32**:4322–4329.

12. Tomizawa H, Yamada H, Hashimoto Y, Imoto T: **Stabilization of lysozyme against irreversible inactivation by alterations of the Asp–Gly sequences.** *Protein Eng* 1995, **8**:1023–1028.

13. Chen H-m, Ford C, Reilly PJ: **Identification and elimination by site-directed mutagenesis of thermolabile aspartyl bonds in *Aspergillus awamori* glucoamylase.** *Protein Eng* 1995, **8**:575–582.

14. Makhatadze GI, Privalov PL: **On the entropy of unfolding.** *Protein Sci* 1996, **5**:507–510.

15. Watanabe K, Masuda T, Ohashi H, Mihara H, Suzuki Y: **Multiple proline substitutions cumulately thermostabilize *Bacillus cerues* ATCC7064 oligo-1,6-glucosidae. Irerefragable proof supporting the proline rule.** *Eur J Biochem* 1994, **226**:277–283.

16. •• Weber PC, Lee S-L, Lewandowski FA, Schadt MC, Chang C-H, Kettner CA: **Kinetic and crystallographic studies of thrombin and ac-(*D*) phe–pro–boro–arg–OH and its lysine, amidine, homolysine and ornithine analogs.** *Biochemistry* 1995, **34**:3750–3757.

Detailed analysis of the effect of entropy on the association constant of thrombin inhibitors. The entropy of more rigid inhibitors in solution is lower

than that of flexible inhibitors. When bound to the enzyme, their entropy is similar. Thus the entropy of a more constrained inhibitor is lower than that of a inhibitor, having more degrees of freedom in solution.

17. Delboni LF, Mande SC, Rentier-Delrue F, Mainfroid V, Turley S, Vellieux FMD, Martial JA, Hol WGL: **Crystal structure of recombinant triose phosphate isomerase from *Bacillus stearothermophilus*. An analysis of potential thermostability factors in six isomerases with known three-dimensional structures points to the importance of hydrophobic interactions.** *Protein Sci* 1995, **4**:2594–2604.

18. Christianson T, Goddette D, Ladin BF, Lau MR, Paech C, Reynolds BB, Wilson CR, Yang S-S: ***Bacillus lentus* alkaline protease with increased stability.** *US Patent* 1994, **5**:340–735.

19. Goddette DW, Christianson T, Ladin BF, Lau M, Mielenz JR, Paech C, Reynolds RB, Yang S-S, Wilson CR: **Strategy and implementation of a system for protein engineering.** *J Biotechnol* 1993, **28**:41–54.

20. • Muir JM, Russell RJM, Hough DW, Danson MJ: **Citrate synthase from the hyperthermophilic Archaeon *Pyrococcus furiosus*.** *Protein Eng* 1995, **8**:583–592.

Analysis of the potential stabilizing effect of sequence differences in two thermophiles relative to the mesophilic homolog. The stabilization in the helical segments seems to augment the analysis of Warren & Petsko [9]. The discussion of the role of glycine residues in α-helix C caps may be very useful in protein stabilization.

21. Perry J, Wetzel R: **Disulfide bonds and thermal stability in T4 lysozyme.** *Science* 1984, **226**:555–557.

22. Byrne MP, Manuel RL, Lowe LG, Stites WE: **Energetic contribution of side chain hydrogen bonding to the stability of staphylococcal nuclease.** *Biochemistry* 1995, **34**:13949–13960.

23. Malcolm BA, Wilson KP, Matthews BW, Kirsch JF, Wilson AC: **Ancestral lysozymes reconstructed, neutrality tested and thermostability linked to hydrocarbon packing.** *Nature* 1990, **345**:86–89.

24. Perutz MF, Raidt H: **Stereochemical basis of heat stability in bacterial ferredoxins and in haemoglobin A2.** *Nature* 1975, **255**:256–259.

25. Szilagyi A, Zavodszky P: **Structural basis for the extreme thermostability of D-glyceraldehyde-3-phosphate from *Thermotoga maritima*: analysis based on homology modelling.** *Protein Eng* 1995, **8**:779–789.

26. Schreiber G, Fersht AR: **Energetics of protein–protein interactions: analysis of the barnase–barstar interface by single mutations and double mutant cycles.** *J Mol Biol* 1995 **248**:478–486.

27. •• Gallagher T, Gilliland G, Wang L, Bryan P: **The prosegment–subtilisin BPN′ complex: crystal structure of a specific 'foldase'.** *Structure* 1995, **3**:907–914.

The three-dimensional structure of the complex of the prosegment–subtilisin BPN′ complex that represents the folding transition state of subtilisin BPN′ formed during its autolytic processing of the mature enzyme. The involvement of helix-sheet interfaces, along with the specific enthalpic stabilization for the complex, suggests that stabilization or destabilization of transition-state folding intermediates may be accomplished with the same set of approaches seen for stabilizing the folded mature enzyme. Also, the role of calcium as a trigger for the autolytic maturation is quite interesting.

28. Alexandrescu AT, Ng YL, Dobsom CM: **Characterization of a trifluoroethanol-induced partial folded state of α-lactalbumin.** *J Mol Biol* 1994, **19**:31–37.

29. Richardson JS, Richardson DC: **Amino acid preferences for specific locations at the ends of α-helices.** *Science* 1988, **240**:1648–1652.

30. Nicholson H, Becktel WJ, Matthews BW: **Enhanced protein thermostability from designed mutations that interact with α-helix dipoles.** *Nature* 1988, **336**:651–656.

31. Doig AJ, Baldwin RL: **N- and C-capping preferences for all 20 amino acids in α-helical peptides.** *Protein Sci* 1995 4:1325–1336.

32. •• Zhang X-J, Baase WA, Shoichet BK, Wilson KP, Matthews BW: **Enhancement of protein stability by the combination of point mutations in T4 lysozyme is additive.** *Protein Eng* 1995, 8:1017–1022.

This represents the combination of a number of variants derived from separate stabilizing approaches. In this paper, the conformational changes in the three-dimensional structures of individual substitutions are found to be combined in the three-dimensional structure of the variant with seven site-specific substitutions.

33. •• Shih P, Kirsch JF: **Design and structural analysis of an engineered thermostable chicken lysozyme.** *Protein Sci* 1995, 4:2063–2072.

Report of the combination of substitutions based on known stabilizing approaches and the 'recruitment' of subtitutions of residues found in more stable homologs.

34. •• Strausberg SL, Alexander PA, Gallagher DT, Gilliland GL, Barnett BL, Bryan PN: **Directed evolution of a subtilisin with calcium-independent stability.** *Biotechnology* 1995, **13**:669–673.

Detailed analysis of the combination of several site-specific variants to recover and then exceed native stability in a variant that has the strong calcium-binding site removed. Protein engineering is used to reduce dependence on calcium for stability by first removing a calcium binding site and then finding compensation substitutions to compensate by introducing additional stability in the vicinity of the deleted calcium bonding site.

35. Wells JA, Powers DB: ***In vitro* formation and stability of engineered disulfide bonds in subtilisin.** *J Biol Chem* 1986, **261**:6564–6570.

36. Mitchinson C, Wells JA: **Protein engineering of disulfide bonds in subtilisin BPN′.** *Biochemistry* 1989, **28**:4807–4815.

37. Frech C, Schmid FX: **Influence of protein conformation on disulfide bond formation in the oxiditative folding of ribonuclease T1.** *J Mol Biol* 1995, **251**:135–149.

38. Erwin CR, Barnett BL, Oliver JD, Sullivan JF: **Effects of engineered salt bridges on the stability of subtilisin BPN′.** *Protein Eng* 1990 4:87–97.

39. Dao-pin S, Sauer U, Nicholson H, Matthews BW: **Contributions of engineered salt bridges to the stability of T4 lysozyme determined by directed mutagenesis.** *Biochemistry* 1991, **30**:7142–7153.

40. •• Wang Y, Shortle D: **The equilibrium folding pathway of staphylococcal nuclease: identification of the most stable chain–chain interactions by NMR and CD spectroscopy.** *Biochemistry* 1995, **34**:15895–15905.

Non-native transition-state structures were found in variants giving folding pathway intermediates. Although the non-native structures were generally localised to one residue, the presence of these structures and their implication for the engineering of kinetic stability may be quite significant.

41. Serrano L, Matouschek A, Fersht AR: **The folding of an enzyme. III. Structure of the transition state for unfolding of barnase analysed by a protein engineering procedure.** *J Mol Biol* 1992, **224**:805–818.

42. Matouschek A, Serrano L, Fersht AR: **The folding of an enzyme. IV. Structure of an intermediate in the refolding of barnase analysed by a protein engineering procedure.** *J Mol Biol* 1992, **224**:819–835.

43. Strausberg S, Alexander P, Wang L, Schwartz F, Bryan P: **Catalysis of a protein folding reaction: thermodynamic and kinetic analysis of subtilisin BPN′ interactions and its propeptide fragment.** *Biochemistry* 1993, **32**:8112–8119.

44. Beasty AM, Hurle M, Manz JT, Stackhouse T, Matthews CR: **Mutagenesis as a Probe of Protein Folding and Stability.** In *Protein Engineering*. Edited by Oxender DL, Fox CF. New York: Alan R Liss; 1987 91–102.

45. Baker D, Agard DA: **Kinetics versus thermodynamics in protein folding.** *Biochemistry* 1994, **33**:7505–7509.