CMLS, Cell. mol. life sci. 53 (1997) 215–223
1420-682X/97/030215-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Stabilization of protein

T. Imoto

Graduate School of Pharmaceutical Sciences, Kyushu University 62, Fukuoka 812-82 (Japan), Fax +81 92 632 3650, e-mail: imoto@imml.phar.kyushu-u.ac.jp

Abstract. The stabilization of proteins is discussed from the theoretical and practical points of view. Methods are described for kinetic stabilization and protection from deterioration, as well as the thermodynamic stabilization of proteins.

Key words. Deterioration; protein; stabilization.

Proteins are maximally evolved to fit the particular physiological conditions in which the protein is acting. Therefore, we can improve the characteristics of proteins according to our new requirements. Due to the physiological requirements, proteins are relatively unstable. We can stabilize them further and use them for longer periods. Moreover, stable proteins can be used at a higher temperature, and we can exploit the higher efficiencies of the catalyses. Thus stabilization is one of the commonest methods used to improve the function of proteins. The stabilization of proteins is a major topic in their use, and there are many excellent reviews on it [1–5].

To understand the stability of proteins we must know about their physical chemistry, especially the forces that participate in their stabilization [6]. Knowledge of the structures of the native and the denatured states of the protein is also required. Due to recent developments in structural biology we know more about the native structure of proteins, but very little about the structure of the denatured state. It has been postulated that a protein retains some residual conformation in the denatured state [7]. In this article, I have presumed that the denaturations are extensive, and I have treated the denatured proteins as more or less completely unfolded entities. To attain maximum stabilization we should avoid any strain in the native state, and the residue to be modified should contribute only minimally to the stability of the parent protein.

In considering the stabilization of proteins, we are usually concerned with thermodynamic stability. In this review, I have also focused on kinetic stabilization and the deterioration of proteins. When irreversible denaturation takes place, we cannot analyze it only in terms of the thermodynamic stabilities. Often we measure the activity under physiological conditions after exposing the protein to more extreme ones. In this case, we are measuring the stability to irreversible denaturation, but not the thermodynamic stability [5].

Thermodynamic stabilization

This aspect has been extensively published and reviewed [1–4]. Therefore, I am only giving a brief review of this subject here. Using this type of stabilization, proteins gain resistance to heat, denaturants, extreme pH and so on, and the qualities of proteins are generally improved.

Theoretical aspects

A protein is in equilibrium between the native (N) and the denatured (D) states. The thermodynamic stabilization concerns only these two states. If the N state is more stable than the D state, then we have more protein molecules in the N state. Therefore, to stabilize a protein, we should enlarge the energy difference (ΔG_D) between the N and the D states (fig. 1). There are two ways to achieve this. One is to lower the energy level of the N state (arrow A in fig. 1) by introducing more favourable interactions stabilizing the proteins in the folded state, or to remove the strains which are apt to operate in the folded structure. The other way is to increase the energy level of the D state (arrow B in fig. 1) by introducing destabilizing effects into the D state.

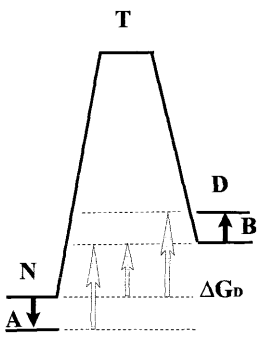


Figure 1. Energy diagrams for thermodynamic stabilization.

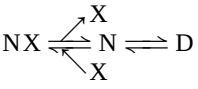
1. Stabilization of the N state

Improving the stabilizing force. (a) Improving the hydrophobic force: The energy of stabilization provided by the transfer of hydrophobic surfaces from the solvent to the interior of a protein is estimated to be 25–30 cal/mol/A^2 [8, 9]. It was calculated that each buried $-CH_2$ contributes an average 1.3 kcal/mol to the conformational stability of the protein [10]. Many examples of this type of stabilization have been reported [11–14]. Improving the hydrophobic interactions in the interior of the protein molecule is more effective [15, 16]. However, to achieve the maximum stabilization some care must be taken to avoid the formation of strains or cavities in the N state. Electrostatic interactions in the interior of a protein could be exchanged with hydrophobic interactions with enhanced stability [17]. The improvement of hydrophobic interactions at the hydrophobic surface of a protein is also effective [3, 18]. However, as will be later discussed, exposing hydrophobic residues at the surface of a protein causes some strains.

(b) Improving hydrogen bonding: The energy of a hydrogen bond is estimated to be 1.1–1.6 kcal/mol [6]. Hydrogen bonds are important in the maintenance of secondary structure, such as α-helices, β-sheets and turns. The N- or C-cap stabilization of the helix by hydrogen bonds is especially significant [19–22]. Hydrogen bonds are particularly important in the buried polar groups [23, 24], and noncharged polar groups that lack hydrogen bonds are very destabilizing ($\Delta\Delta G >= 3$ kcal/mol, [2]). Sometimes, the incorporation of fixed water molecules provides relief from this strain [25]. Stabilization by improving the hydrogen bond has been reported for barnase [11], subtilisin [26], and T4 lysozyme [27].

(c) Improving electrostatic interaction: The strength of a salt bridge is sensitive to geometry, distance and the environment (such as exposure and the surrounding charges), and the contribution to the stability is estimated to be 0 to a few kcal/mol [28]. Improving an electrostatic interaction around the helix dipole is another concern [19, 20, 29].

(d) Complexation: By the formation of a stable complex, we can reduce the population of the protein in the D state according to the following equilibrium:



Improving the ligand binding site is one way of doing this. A preferential binding of a ligand to the folded state of the protein usually enhances the stability of the protein [30–34]. The de novo design of the metal-binding site is the first choice for this sort of stabilization and has been previously reviewed [35, 36]. The introduction of a calcium-binding loop into human lysozyme greatly enhanced its stability [37]. Oligomerizations are another way for the protein to acquire stabilizations and have also been reviewed [38]. To stabilize the oligomerizing forces at the subunit contact surface is one way to improve the association phenomenon [39–41]. The introduction of an –SS– bridge between subunits [42–46] or the connection of the subunits into one protein (single chain) [3, 47, 48] is a more effective way to stabilize the subunit interaction.

Release from strain. (a) Release from steric strain: Ramachandran plots of folded proteins rationally showed us that many geometrical strains are evoked by the folding of proteins. These strains can be released by structural changes in the protein [49]. Deletion or insertion of amino acids or the substitution of certain residues by glycine [33, 51–53] is effective, while an introduction of glycine leads to some destabilization as will be later discussed.

(b) Release from electrostatic strain: An electrostatic strain is easily evoked by placing similar charges close together or just by burying a charge in a hydrophobic environment [54]. Acidic groups showing abnormally high pK_a or basic groups showing abnormally low pK_a are destabilizing to the protein, and the removal of these charges leads to increased stabilization [55].

(c) Removal of cavity: The cavity in the hydrophobic box in a protein lowers the stability of the protein by 20 cal/mol/$Å^2$ [56] due to the loss of van der Waals interactions. Filling the cavity leads to further stabilization [57–60].

(d) Removal of exposed hydrophobic residue: Placing a hydrophobic residue at the surface of a protein is energetically unfavourable and a stabilization of 2.2 kcal/mol was obtained by replacing the exposed hydrophobic residue (Tyr26) by a polar one (Cys) in lambda Cro [61].

2. Destabilization of the D state

The D state is greatly stabilized by the flexibility of the chain (chain entropy). If we restrict the flexibility of the chain, the D state looses its entropy, and it is destabilized.

Introduction of cross link. A cross link greatly restricts the movement of the chain in the D state. The relationship between the position of the cross link and the entropy loss can be calculated [62, 63]. Chemical cross-linking is one way to achieve stabilization [64, 65]. It is important to introduce the cross link using a reagent with an appropriate length, at the residues which have contributed little to the other stabilizing interactions. An introduction of a new –SS– bond is another method [66–73] and was previously reviewed [74]. The introduction of any strain in the folded structure should be avoided for effective stabilization by cross-linking.

Introduction of proline. A proline residue brings a five-membered ring into the peptide chain and causes

restriction of the movement of the unfolded protein molecule. The entropy loss for the unfolded state of the protein caused by the introduction of a proline residue was estimated to be 4 cal/deg/mol [75]. The effects of various mutations were tested [76–82]. The introduction of a proline residue is apt to cause a strain in the folded state [83], especially at the point of the mutation and at the N-terminal residue because the five-membered ring includes an N-terminal amide nitrogen. In this sense, the introduction of the Gly-Pro sequence was recommended because the structurally flexible glycine at the N-terminal of the proline reduced this strain [50, 51].

Removal of glycine. A glycine residue is devoid of β-carbon and has greater configurational flexibility than the other residues which have β-carbon. The entropy loss for the unfolded state by the substitution of glycine to alanine was estimated to be 2.4 cal/deg/mol [75], and this method of substitution has effectively stabilized the proteins [81, 82]. However, glycine residues are apt to be placed at conformationally severe positions and the substitutions often cause strains in the folded states. Moreover, larger residues have more entropy in the unfolded state, and the effect of the substitution was easily canceled [84].

3. Other methods

Glycosylation or modification with synthetic polymer. It is well known that when we express proteins in certain cells, we can often glycosylate them by introducing the consensus sequence for N-glycosylation (AsnXaaThr or Ser). The proteins yielded were usually more stable [85–90]. Chemical modification with synthetic polymers can also stabilize proteins. By modification with polyethylene glycol, the half-life of a protein was increased in vivo [91].

Immobilization. Immobilizations are occasionally employed in protein engineering, and it was found that proteins became more stable in the immobilized state [92–95].

Practical aspects

Because of the physiological requirements, proteins are so fragile that I suspect nature has 'willingly' destabilized them, or at least paid little attention to their stabilization. It is also known that stabilization is sacrificed for the sake of producing effective functional environments [55, 96]. On the other hand, we know about many extremely stable proteins that are composed of constituents similar to normal proteins [94, 97]. Thus, we can stabilize proteins by improving the stabilizing effects previously mentioned. The stabilizing operations are usually universal and we can apply the method used by one protein to others. Moreover, the stabilizing operations are additive [3, 82, 98–100], and we can accumulate the effects [101–104]. Only when the second stabilizing operation is introduced very close to the first one, or when two operations are in a cooperative site, does the additivity become unpredictable. Sometimes, cooperative mutations caused an effective stabilization [105].

Table 1. Energy for Gly-Pro or Pro-Gly mutants of lysozyme [50].

Lysozyme	Residue No. 100 101 102 103	ΔG_D (kcal/mol)	Strain in N (kcal/mol)
Rabbit	-Ser-Asp-Pro-Gln-		
Hen	-Ser-Asp-Gly-Asn-	10.6	
DP	-Pro-	9.8	2.0
PG	-Pro-	7.5	3.6
GP	-Gly-Pro-	12.1	0.2

Random modifications. Random mutations are a good method to improve the stability of proteins. When we do not have structural information, this is the only method to be employed. The key point with this method is to select the stabilized mutant effectively by assaying the function after exposing under or at an extreme condition [26]. It is also effective to select revertants after a destabilizing mutation. In this case, a biological positive selection can be conducted using bacteria, including thermophilic bacteria, dependent on the protein [106, 107]. We can improve the quality by accumulating random mutations [108]. After we obtain information by random mutation about the residue that is responsible for the stability, we can apply site-directed mutageneses on the residue to improve the effect further.

Site-directed modifications. As previously mentioned, we have acquired a number of methods to improve the stability of proteins, and these can be applied by site-directed modification [2]. Examples of the modifications were mentioned in the previous section.

Learning form nature. Strategies can be learnt from the protein working under extreme conditions [33, 38, 109, 110]. Sorting the residue that is responsible for the stabilization of the protein by comparing the stabilities and the primary structures of the proteins from different origins (comparative biochemistry) is another method. We compared the stabilities and the amino acid sequences of lysozymes from different origins and found that rabbit lysozyme is more stable than hen or human lysozyme [111]. Rabbit lysozyme has more proline residues (4) than hen (2) or human (2) lysozyme, and this was estimated to be the reason for the stability. We tried to improve the stability in hen lysozyme by introducing a proline residue at position 102 where the rabbit lysozyme has a proline residue. As previously mentioned, the introduction of a proline residue leads to the stabilization of the protein, but a proline residue has a five-membered ring, and its introduction easily causes a strain in the N state of the protein. We thought this position might be a good one because it is a

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3[X]} P$$

$$v = \frac{k_1 k_3[N][X]}{k_2 + k_3[X]} \; ; \; K_D = \frac{k_1}{k_2}$$

$$k_2 >> k_3[X] \; ; \; v = k_3 K_D[N][X]$$

$$k_2 << k_3[X] \; ; \; v = k_1[N]$$

$$\Delta G = - RT \ln K_D$$

$$\Delta G^{\ddagger} = -RT \ln \frac{k_1 h}{k_B T}$$

Figure 2. Kinetic equations for irreversible denaturation coupled with the denatured state.

naturally occurring position. However, after several trials (table 1), we succeeded in attaining stabilization by introducing a Gly-Pro sequence [50, 51]. A proline residue forms the ring with the N-terminal amide nitrogen, and only when the N-terminal amino acid is a glycine could the proline residue be situated without causing strain in the N state (table 1). In this example, we obtained a hint on how to improve the stability. It is noteworthy that considerable strains were introduced by the Pro-Gly sequences compared to the Gly-Pro sequence.

Kinetic stabilization

Sometimes proteases are not stabilized against autolysis even after thermodynamic stabilization [112]. This is because protease digestion usually proceeds via the D state of the substrate protein [113]. A kinetic stabilization is required for protection against irreversible denaturation coupled with the D state of the protein. This sort of consideration is of supreme importance in using proteins as medicines.

Theoretical aspects

Irreversible denaturation coupled with the D state can be analyzed as shown in fig. 2. The [X] can be a concentration of protease, chemical reagent, denatured protein (in the case of aggregation), and so on. As long as $k_3[X]$ is much smaller than k_2, the thermodynamic stabilization is effective. However, when $k_3[X]$ becomes much larger than k_2, as is the case where the protease concentration ([X]) is very high, only k_1 (unfolding rate constant) is effective for the denaturation rate. Therefore, a kinetic stabilization (decreasing k_1) becomes important for irreversible denaturation coupled with the D state instead of a thermodynamic one [114]. We are interested in the ratio of k_1 and k_2 (i.e. K_D) for the thermodynamic stability and in only k_1 for the kinetic stability.

Practical aspects

Irreversible denaturations coupled with the D states, such as protease digestions or some forms of chemical

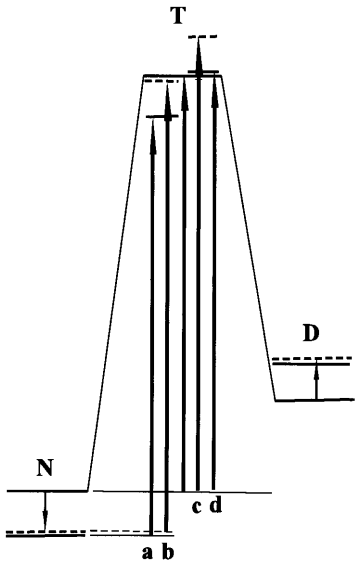


Figure 3. Energy diagrams for kinetic stabilization.

reactions, can be effectively resisted only by decreasing k_1 or increasing the delta activation free energy for the unfolding. Sometimes we can increase the delta activation free energy for the unfolding by thermodynamic stabilization. First, we consider thermodynamic stabilization by stabilizing the N state. If the modified region of the protein holds its folded structure in the transition state of the unfolding, the stabilizing effect is brought to the transition state and the delta activation free energy for the unfolding increases little (fig. 3 arrow a). On the other hand, if the region loses its folded structure in the transition state of the unfolding, the stabilization effect is lost in the transition state, and the delta activation free energy for the unfolding increases accordingly (fig. 3 arrow b). This means that we should stabilize the N state where the protein is disordered in the transition state of the unfolding to obtain a kinetic stabilization as well. Second, we consider thermodynamic stabilization by destabilizing the D state. In this case, the entropy loss in the disordered structure is important. If the stabilization effect is introduced where the protein is disordered in the transition state of the unfolding, the destabilization effect is brought about in this state also, and the delta activation free energy for the unfolding could be increased (fig. 3 arrow c), otherwise it does not (fig. 3 arrow d). In conclusion, if we introduce a thermodynamic stabilization where the protein is disordered at the transition state for the unfolding, we can attain a kinetic stabilization as well as a thermodynamic one.

For kinetic stabilization, it is important to predict the site where the protein is largely disordered in the transition state for the unfolding. This site can be predicted from the results of stabilization at that site, whether or not kinetic stabilization was also attained. A practical method to predict the site was presented by Fersht et al. [115]. We also developed a convenient method to

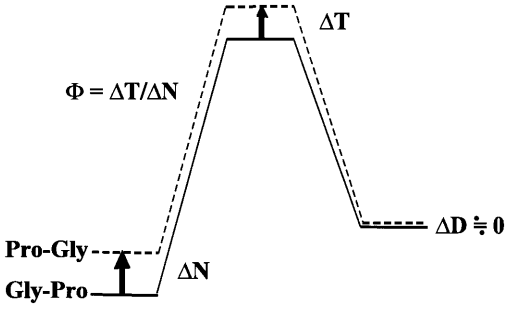


Figure 4. Energy diagrams for the Pro-Gly and Gly-Pro mutants.

predict the site [116]. As previously mentioned, the Pro-Gly sequence introduced much more strain in the N state than the Gly-Pro sequence, while their energy levels for the D states are similar. We introduced the Gly-Pro or Pro-Gly sequence at the same site and determined how much strain introduced into the N state by the Pro-Gly sequence was brought about in the transition state. This amount (Φ) is proportional to the degree of retention of the native structure at the site in the transition state of the unfolding (fig. 4). We determined the Φ values for several sites of lysozyme (fig. 5). If we stabilize a protein at the site with a small Φ value, a kinetic stabilization is also attainable.

Protection from deterioration

Thanks to the development of protein engineering we can utilize proteins in various fields, such as medicine, industry, and so on. The deterioration of proteins [117], irreversible chemical reactions [118] and irreversible denaturations are particular problems in the prolonged usage or storage of proteins under severe conditions. We elucidated that the deterioration of proteins can be simulated in a short time by heating the sample at 100 °C [119]. By heating at pH 4 and 100 °C, lysozyme lost its activity following pseudo first-order kinetics ($k = 0.70\ h^{-1}$) after a minor heat activation. The destabilized protein where the 6-127 disulphide was reduced and carboxyamidated lost activity faster ($k = 1.59\ h^{-1}$) than the unmodified protein. On the other hand, the stabilized protein where 1–15 was chemically cross-linked lost activity more slowly ($k = 0.59\ h^{-1}$). There was a good correlation between stability and inactivation. The reasons for the inactivation were: 1. Isomerization at the Asp residue. 2. Deamidation of the Asn residue. 3. Racemization of the Asx residue. 4. Hydrolysis of the Asp-Xaa bond. 5. Interchange of the SS bond. 6. Destruction of the Cys residue. The protein gradually becomes unstable by the accumulation of these chemical reactions. Finally, it becomes so unstable that it cannot hold its folded structure under physiological conditions and loses its activity [119]. This can be understood from the previous result that the inactivation was closely correlated with the thermodynamic stability of the protein. Reactions 1, 2, and 3 predominantly occurred at the Asx-Gly sequences. Reactions 2 and 3 were accelerated in the presence of a phosphate ion [120]. Rearrangements of –SS– bonds were also active at pH 6, and this reaction was depressed by the addition of copper ion [121]. It was found that trifluoroethanol

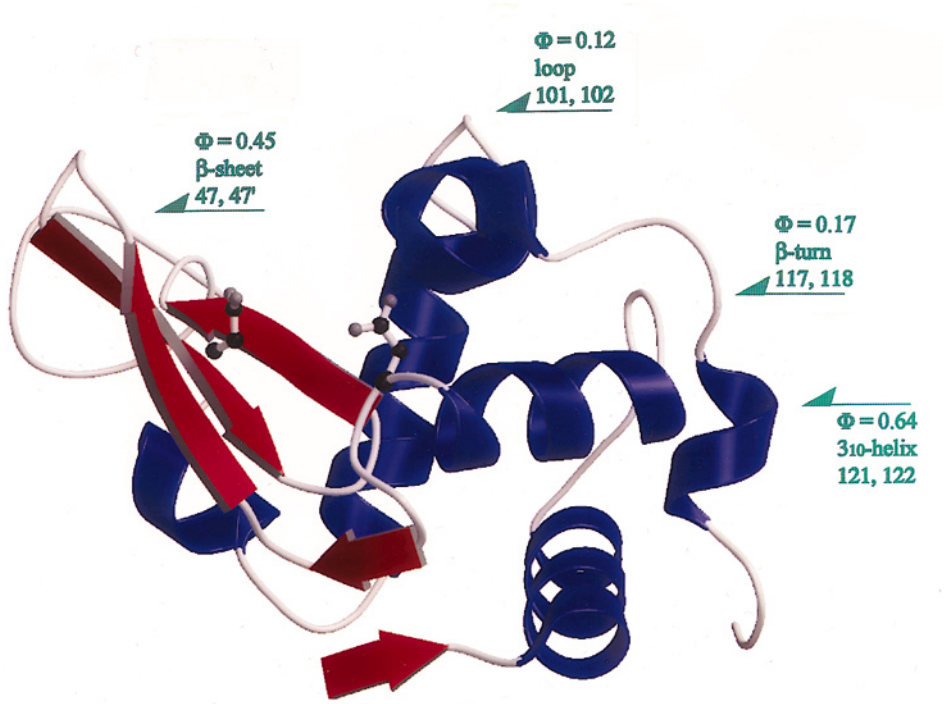


Figure 5. The degrees of retention of native conformation (Φ values) were determined by employing Gly-Pro and Pro-Gly mutants at various sites of lysozyme [116]. MOLSCRIPT program [124] was employed for the presentation of the model of lysozyme.

dramatically depressed the deterioration [120]. Asp-Gly sequences are very susceptible to chemical reaction, and the deterioration was greatly depressed by the mutation of Asp-Gly to Asp-Ala [122].

To prevent the deterioration, the chemical reactions should be depressed by avoiding phosphate ions, adding trifluoroethanol, avoiding alkaline conditions, and adding copper ion. Deleting Asx-Gly sequences is also effective. These chemical reactions usually occur in the D state of the proteins, and these reactions can be depressed by the thermodynamic stabilization of the proteins. The stabilized proteins can keep their folded structure even after chemical modifications. Thus, thermodynamic stabilization can prevent deterioration in two ways.

I have described mainly the proteins that readily refold reversibly from the unfolded state. However, many proteins are irreversibly denatured and tend to form aggregates, especially at moderately high concentrations. To protect against this kind of deterioration [117], we can thermodynamically stabilize proteins by glycosylation, immobilization, and so on. Additions of some sort of stabilizer, such as 'salting-in' additives, are also effective [117].

Conclusions

We can stabilize proteins thermodynamically or kinetically or against deterioration. We should choose a stabilization method appropriate to our purpose. Biological functions are apt to be coupled to the fluctuations of the protein molecules [123], and conflicting effects on stabilizations and fluctuations will be considered in the future.

1 Shortle D. (1992) Mutational studies of protein structures and their stabilities. Quarterly Reviews of Biophysics **25:** 205–250
2 Matthews B. W. (1995) Studies on protein stability with T4 lysozyme. Adv. Protein Chem. **46:** 249–278
3 Terwilliger T. C. (1995) Engineering the stability and function of gene V protein. Adv. Protein Chem. **46:** 177–215
4 Serrano L., Kellis J. T. Jr., Cann P., Matouschek A. and 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
5 Shaw A. and Bott R. (1996) Engineering enzyme for stability. Current Opinion Struct. Biol. **6:** 546–550
6 Pace C. N., Shirley B. A., Mcnutt M. and Gajiwala K. (1996) Forces contributing to the conformational stability of proteins. FASEB J. **10:** 75–83
7 Shortle D. (1996) The denatured state (the other half of the folding equation) and its role in protein stability. FASEB J. **10:** 27–34
8 Chothia C. (1974) Hydrophobic bonding and accessible surface area in proteins. Nature **248:** 338–339
9 Richard F. M. (1977) Areas, volumes, packing and protein strucure. Annu. Rev. Biophys. Bioeng. **6:** 151–176
10 Pace C. N. (1992) Contribution of the hydrophobic effect to globular protein stability. J. Mol. Biol. **226:** 29–35
11 Kellis Jr. J. T., Nyberg K., Sali D. and Fersht A. R. (1988) Contribution of hydrophobic interactions to protein stability. Nature **333:** 784–786
12 Matsumura M., Becktel W. J. and Matthews B. W. (1988) Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile3. Nature **334:** 406–410
13 Yutani K., Ogasahara K., Tsujita T. and Sugino Y. (1987) Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit. Proc. Natl. Acad. Sci. USA **84:** 4441–4444
14 Kellis Jr. J. T., Nyberg K. and Fersht A. R. (1989) Energetics of complementary sidechain packing in a protein hydrophobic core. Biochemistry **28:** 4914–4922
15 Yutani K., Ogasahara K., Sugino Y. and Matsushiro A. (1977) Effect of a single amino acid substitution on stability of conformation of a protein. Nature **267:** 274–275
16 Inoue M., Yamada H., Yasukochi T., Kuroki R., Miki T., Horiuchi T. et al. (1992) Multiple role of hydrophobicity of tryptophan-108 in chicken lysozyme: structural stability, saccharide binding ability, and abnormal pK_a of glutamic acid-35. Biochemistry **31:** 5545–5553
17 Waldburger C. D., Schildbach J. F. and Sauer R. T. (1995) Are buried salt bridges important for protein stability and conformational specificity? Nature Struct. Biol. **2:** 122–128
18 Van den Burg B., Dijkstra B. W., Vriend G., Van der Vinne B., Venema G. and Eijsink V. G. (1994) Protein stabilization by hydrophobic interactions at the surface. Eur. J. Biochem. **220:** 981–985
19 Scholtz J. M., Qian H., Robbins V. H. and Baldwin R. L. (1993) The energetics of ion-pair and hydrogen-bonding interactions in a helical peptide. Biochemistry **32:** 9668–9676
20 Bordo D. and Argos P. (1994) The role of side-chain hydrogen bonds in the formation and stabilization of secondary structure in soluble proteins. J. Mol. Biol. **243:** 504–519
21 Zhukovsky E. A., Mulkerrin M. G. and Presta L. G. (1994) Contribution to global protein stabilization of the N-capping box in human growth hormone. Biochemistry **33:** 9856–9864
22 Petukhov M., Yumoto N., Murase S., Onmura R. and Yoshikawa S. (1996) Factors that affect the stabilization of alpha-helices in short peptides by a capping box. Biochemistry **35:** 387–397
23 Chen Y. W., Fersht A. R. and Henrick K. (1993) Contribution of buried hydrogen bonds to protein stability. The crystal structures of two barnase mutants. J. Mol. Biol. **234:** 1158–1170
24 Blaber M., Lindstrom J. D., Gassner N., Xu J., Heinz D. W. and Matthews B. W. (1993) Energetic cost and structural consequences of burying a hydroxyl group within the core of a protein determined from Ala→Ser and Val→Thr substitutions in T4 lysozyme. Biochemistry **32:** 11363–11373
25 Berghuis A. M., Guillemette J. G., McLendon G., Sherman F., Smith M. and Brayer G. D. (1994) The role of a conserved internal water molecule and its associated hydrogen bond network in cytochrome c. J. Mol. Biol. **236:** 786–799
26 Bryan E. P., Rollence M. L., Pantoliano M. W., Wood J., Finzel B. C., Gilliland G. L. et al. (1986) Protease of enhanced stability: characterization of a thermostable variant of subtilisin. Proteins **1:** 326–334
27 Alber T., Dao-pin S., Wilson K., Wozniak J. A., Cook S. P. and Matthews B. W. (1987) Contributions of hydrogen bonds of Thr157 to the thermodynamic stability of phage T4 lysozyme. Nature **330:** 41–46
28 Horovitz A., Serrano L., Avron B. and Fersht A. R. (1990) Strength and co-operativity of contributions of surface salt bridges to protein stability. J. Mol. Biol. **216:** 1031–1044
29 Nicholson N., Becktel W. J. and Matthews B. W. (1988) Enhanced protein thermostability from designed mutations that interact with alpha-helix dipoles. Nature **336:** 651–656
30 Denisov I. G. (1992) Thermal stability of proteins in intermolecular complexes. Biophy. Chem. **44:** 71–75
31 Jaenicke R. (1987) Folding and association of proteins. Progr. Biophys. Mol. Biol. **49:** 117–237
32 Rise B., Stempfer G., Rudolph R. and Jaenicke R. (1992) Stability and characterization of the stabilizing effect of point mutations of pyruvate oxidase from Lact. plantarum. Protein Sci. **1:** 1699–1718

33 Arnold F. H. (1993) Engineering proteins for nonnatural environments. FASEB J. **7:** 744–749

34 Risse B., Stempfer G., Rudolph R., Schumacher G. and Jaenicke R. (1992) Characterization of the stabilizing effect of point mutations of pyruvate oxidase from Lactobacillus plantarum: protection of the native state by modulating coenzyme binding and subunit interaction. Protein Sci. **1:** 1710–1718

35 Matthews D. J. (1995) Interfacial metal-binding site design. Current Opinion Biotech. **6:** 419–424

36 Regan I. (1995) Protein design: novel metal-binding sites. Trends Biochem. Sci. **20:** 280–285

37 Kuroki R., Taniyama Y., Seko C., Nakamura H., Kikuchi M. and Ikehara M. (1989) Design and creation of a Ca^{++} binding site in human lysozyme to enhance structural stability. Proc. Natl. Acad. Sci. USA **86:** 6903–6907

38 Ruvinov S. B. and Miles E. W. (1994) Thermal inactivation of tryptophan synthase. Stabilization by protein-protein interaction and protein-ligand interaction. J. Biol. Chem. **269:** 11703–11706

39 Hendsch Z. S., Jonsson T., Saure R. T. and Tidor B. (1996) Protein stabilization by removal of unsatisfied polar groups: computational approaches and experimental tests. Biochemistry **35:** 7621–7625

40 Kirino H., Aoki M., Aoshima M., Hayashi Y., Ohba M., Yamagishi A. et al. (1994) Hydrophobic interaction at the subunit interface contributes to the thermostability of 3-isopropylmalate dehydrogenase from an extreme thermophile, Thermus thermophilus. Eur. J. Biochem. **220:** 275–281

41 Kawamura S., Kakuta Y., Tanaka I., Hikichi K., Kuhara S., Yamasaki N. et al. (1996) Glycine-15 in the bend between two alpha-helices can explain the thermostability of DNA binding protein HU from Bacillus stearothermophilus. Biochemistry **35:** 1195–1200

42 Tamura A., Kojima S., Miura K. and Sturtevant J. M. (1994) Effect of an intersubunit disulfide bond on the stability of Streptomyces subtilisin inhibitor. Biochemistry **33:** 14512–14520

43 Reiter Y., Brinkmann U., Jung S. H., Lee B., Kasprzyk P. G., King C. R. et al. (1994) Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide stabilization of the Fv fragment. J. Biol. Chem. **269:** 18327–18331

44 Reiter Y., Brinkmann U., Webber K. O., Jung S. H., Lee B. and Pastan I. (1994) Engineering interchain disulfide bonds into conserved framework regions of Fv fragments: improved biochemical characteristics of recombinant immunotoxins containing disulfide-stabilized Fv. Protein Eng. **7:** 697–704

45 Sorokin A., Lemmon M. A., Ullrich A. and Schlessinger J. (1994) Stabilization of an active dimeric form of the epidermal growth factor receptor by introduction of an inter-receptor disulfide bond. J. Biol. Chem. **269:** 9752–9759

46 Gokhale R. S., Agarwalla S., Francis V. S., Santi D. V. and Balaram P. (1994) Thermal stabilization of thymidylate synthase by engineering two disulfide bridges across the dimer interface. J. Mol. Biol. **235:** 89–94

47 Bird R. E., Hardman K. D., Jacobson J. W., Johnson S., Kaufman B. M., Lee S. M. et al. (1988) Single-chain antigen-binding proteins. Science **242:** 423–426

48 Huston J. S., Levinson D., Mudgett-Hunter M., Tai M. S., Novotny J., Margolies M. N. et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc. Natl. Acad. Sci. USA **85:** 5879–5883

49 Hodel A., Kautz R. A. and Fox R. O. (1995) Stabilization of a strained protein loop conformation through protein engineering. Protein Sci. **4:** 484–495

50 Ueda T., Tamura T., Maeda Y., Hashimoto Y., Miki T., Yamada H. et al. (1993) Stabilization of lysozyme by the introduction of Gly-Pro sequence. Protein Eng. **6:** 183–187

51 Motoshima M., Ueda T., Hashimoto Y., Tsutsumi M. and Imoto T. (1995) Correlation between the differences in the free energy change and conformational energy in the folded state of hen lysozyme with Gly-Pro and Pro-Gly sequences introduced to the same site. J. Biochem. **118:** 1138–1144

52 Kimura S., Kanaya S. and Nakamura H. (1992) Thermostabilization of Escherichia coli ribonuclease HI by replacing left-handed helical Lys95 with Gly or Asn. J. Biol. Chem. **267:** 22014–22017

53 Sites W. E., Gittis A. G., Lattman E. E. and Shortle D. (1991) In a staphylococcal nuclease mutant the side-chain of a lysine replacing valine 66 is fully buried in the hydrophobic core. J. Mol. Biol. **221:** 7–14

54 Inoue M., Yamada H., Hashimoto Y., Yasukochi T., Hamaguchi K., Miki T. et al. (1992) Stabilization of a protein by removal of unfavorable abnormal pKa: substitution of undissociable residue for glutamic acid-35 in chicken lysozyme. Biochemistry **31:** 8816–8821

55 Matthews B. W. (1996) Structural and genetic analysis of the folding and function of T4 lysozyme. FASEB J. **10:** 35–41

56 Yamada H., Kanaya E., Inaka K., Ueno Y., Ikehara M. and Kikuchi M. (1994) Stabilization and enhanced enzymatic activities of a mutant human lysozyme C77/95A with a cavity space by amino acid substitution. Biol. Pharm. Bull. **17:** 192–196

57 Ishikawa K., Nakamura H., Morikawa K. and Kanaya S. (1993) Stabilization of Escherichia coli ribonuclease HI by cavity-filling mutations within a hydrophobic core. Biochemistry **32:** 6171–6178

58 Karpusas M., Baase W. A., Matsumura M. and Matthews B. W. (1989) Hydrophobic packing in T4 lysozyme probed by cavity-filling mutants. Proc. Natl. Acad. Sci. USA **86:** 8237–8241

59 Baldwin E., Xu J., Hajiseyedjavadi O., Baase W. A. and Matthews B. W. (1996) Thermodynamic and structural compensation in ''size-switch'' core repacking variants of bacteriophage T4 lysozyme. J. Miol. Biol. **259:** 542–559

60 Pakula A. A. and Sauer R. T. (1990) Reverse hydrophobic effects relieved by amino-acid substitutions at a protein surface. Nature **344:** 363–364

61 Kin S. H., Konishi Y., Denton M. E. and Scheraga H. A. (1984) Influence of an extrinsic cross-link on the folding pathway of ribonucrease A. Conformational and thermodynamic analysis of cross-linked (lysine7-lysine41)-ribonuclease A. Biochemistry **23:** 5504–5512

62 Poland D. G. and Scheraga H. A (1965) Statistical mechanics of noncovalent bonds in polyamino acids VIII. Covalent loops in proteins. Biopolymers **3:** 379–399

63 Ueda T., Yamada H., Hirata M. and Imoto T. (1985) An intramolecular cross-linkage of lysozyme. Formation of cross-links between lysine-1 and histidine-15 with bis(bromoacetamide) derivatives by a two-stage reaction procedure and properties of the resulting derivatives. Biochemistry **24:** 6316–6322

64 Ueda T., Yamada H., Sakamoto N., Abe Y., Kawano K., Terada Y. et al. (1991) Preparation and properties of a lysozyme derivative in which two domains are cross-linked intramolecullary between Trp62 and Asp101. J. Biochem. **110:** 719–725

65 Yamada H., Kuroki R., Hirata M. and Imoto T. (1983) An intramolecular cross-linkage of lysozyme. Imidazole catalysis of the formation of the cross-link between lysine 13 (ε-amino) and leucine 129 (α-carboxyl) by carbodiimide reaction. Biochemistry **22:** 4551–4556

66 Matsumura M., Becktel W. J., Levitt M. and Matthews B. W. (1989) Stabilization of phage T4 lysozyme by engineered disulfide bonds. Proc. Natl. Acad. Sci. USA **86:** 6562–6556

67 Matsumura M., Signor G. and Matthews B. W. (1989) Substantial increase of protein stability by multiple disulphide bonds. Nature **342:** 291–293

68 Aberle A. M., Reddy H. K., Heeb N. V. and Nambiar K. P. (1994) Stabilization of beta-ribbon structure in peptides using disulfide bonds. Biochem. Biophys. Res. Commun. **200:** 102–107

69 Kanaya S., Katsuda C., Kimura S., Nakai T., Kitakuni E., Nakamura H. et al. (1991) Stabilization of Escherichia coli ribonuclease H by introduction of an artificial disulfide bond. J. Biol. Chem. **266:** 6038–6044

70 Wetzel R., Perry L. J., Baase W. A. and Becktel W. J. (1988) Disulfide bonds and thermal stability in T4 lysozyme. Proc. Natl. Acad. Sci. USA **85:** 401–405
71 Betz S. E. (1993) Disulfide bonds and the stability of globular proteins. Protein Sci. **2:** 1551–1558
72 Clarke J., Henrick K. and Fersht A. R. (1995) Disulfide mutants of barnase I: changes in stability and structure assessed by biophysical methods and X-ray crystallography. J. Mol. Biol. **253:** 493–504
73 Hinck A. P., Truckses D. M. and Markley J. L. (1996) Engineered disulfide bonds in staphylococcal nuclease: effects on the stability and conformation of the folded protein. Biochemistry **35:** 10328–10338
74 Matthews B. W., Nicholson H. and Becktel W. J. (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. Proc. Natl. Acad. Sci. USA **84:** 6663–6667
75 Hardy F., Vriend G., Veltman O. R., van der Vinne B., Venema G. and Eijsink V. G. (1993) Stabilization of Bacillus stearothermophilus neutral protease by introduction of prolines. FEBS Lett. **317:** 89–92
76 Eisenberg D. and McLachlan A. D. (1986) Solvation energy in protein folding and binding. Nature **319:** 199–203
77 Ooi T., Oobatake M., Nemethy G. and Scheraga H. A. (1987) Accessible surface area as a measure of the thermodynamic parameters of hydration of peptides. Proc. Natl. Acad. Sci. USA **84:** 3086–3090
78 Ooi T. and Oobatake M. (1988) Effects of hydrated water on protein folding. J. Biochem. **103:** 114–120
79 Watanabe K., Kitamura K. and Suzuki Y. (1996) Analysis of the critical sites for protein thermostabilization by proline substitution in oligo-1,6-glucosidase from Bacillus coagulns ATCC7050 and the evolutionary consideration of proline residues. App. Env. Microbiol. **62:** 2066–2073
80 Margarit I., Campagnoli S., Frigerio F., Grandi G., De Filippis V. and Fontana A. (1992) Cumulative stabilizing effects of glycine to alanine substitutions in Bacillus subtilis neutral protease. Protein Eng. **5:** 543–550
81 Margarit I., Campagnoli S., Frigerio F., Grandi G., De Filippis V. and Fontana A. (1992) Cumulative stabilizing effects of glycine to alanine substitutions in Bacillus subtilis neutral protease. Protein Eng. **5:** 543–550
82 Dixon M. M., Nicholson H., Shewchuk L., Baase W. A. and Matthews B. W. (1992) Structure of a hinge-bending bacteriophage T4 lysozyme mutant, Ile3→Pro. J. Mol. Biol. **227:** 917–933
83 Gray T. M. and Matthews B. W. (1987) Structural analysis of the temperature-sensitive mutant of bacteriophage T4 lysozyme, glycine156-aspartic acid. J. Biol. Chem. **262:** 16858–16864
84 Mer G., Hietter H. and Lefevre J. V. (1996) Stabilization of proteins by glycosylation examined by NMR analysis of a fucosylated proteinase inhibitor. Nature Struct. Biol. **3:** 45–53
85 Ueda T., Iwashita H., Hashimoto Y. and Imoto T. (1996) Stabilization of lysozyme by introducing N-glycosylation signal sequence. J. Biochem. **119:** 157–161
86 Kern G., Schulke N., Schmid F. X. and Jaenicke R. (1992) Quaternary structure and stability of internal, external and core-glycosylated invertase from yeast. Protein Sci. **1:** 120–131
87 Kern G., Kern D., Jaenicke R. and Seckler R. (1993) Kinetics of folding and association of differently glycosylated variants of invertase from Sacch. cerevisiae. Protein Sci. **2:** 1862–1868
88 Turkova J., Vohnik S., Helusova S., Benes M. J. and Ticha M. (1992) Galactosylation as a tool for the stabilization and immobilization of proteins. J. Chromatog. **597:** 19–27
89 Wang C. O., Eufemi M., Turano C. and Giartosio A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. Biochemistry **35:** 7299–7307
90 Kuan C. T., Wang Q. C. and Pastan I. (1994) Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. J. Biol. Chem. **269:** 7610–7616
91 Jafri F., Husain S. and Saleemuddin M. (1993) Immobilization and stabilization of invertase using specific polyclonal antibodies. Biotech. App. Biochem. **18:** 401–408
92 Asther M. and Meunier J. C. (1993) Immobilization as a tool for the stabilization of lignin peroxidase produced by Phanerochaete chrysosporium INA-12. App. Biochem. Biotech. **38:** 57–67
93 Liagre B., Battu S., Rigaud M. and Beneytout J. L. (1996) Effectiveness of talc as adsorbent for stabilization and expression of Pisum sativum hortense cv solara lipoxygenase-lyase coupled activities. J. Agric. Food Chem. **44:** 2057–2062
94 Bainchi D., Golini P., Bortolo R. and Cesti P. (1996) Immobilization of penicillin G acylase on aminoalkylated polyacrylic supports. Enz. Microbial Tech. **18:** 592–596
95 Jaenicke R. (1996) Stability and folding of ultrastable proteins: eye lens crystallins and enzymes from thermophiles. FASEB J. **10:** 84–92
96 Shoichet B. K., Baase W. A., Kuroki R. and Matthews B. W. (1995) A relationship between protein stability and protein function. Proc. Natl. Acad. Sci. USA **92:** 452–456
97 Jaenicke R. (1991) Protein stability and molecular adaptation to extreme conditions. Eur. J. Biochem. **202:** 715–728
98 Shortle D. and Meeker A. K. (1986) Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation. Proteins **1:** 81–89
99 Strausberg S. L., Alexander P. A., Gallagher D. T., Gilliland G. L., Barnett B. L. and Bryan P. N. (1995) Directed evolution of a subtilisin with calcium-independent stability. Biotchnology **13:** 669–673
100 Shih P. and Kirsch J. F. (1995) Design and structural analysis of an engineered thermostable chicken lysozyme. Protein Sci. **4:** 2068–2072
101 Wels, J. A. (1990) Additivity of mutational effects in proteins. Biochemistry **29:** 8509–8517
102 Wong C.-H., Chen S.-T., Hennen W. J., Bibbs J. A., Wang Y.-F., Lin J. L.-C. et al. (1990) Enzymes in organic synthesis: Use of subtilisin and a highly stable mutant derived from multiple site-specific mutations. J. Am. Chem. Soc. **112:** 945–953
103 Pantoliano M. W., Whitlow M., Wood J. E., Dodd S. W., Hardman K. D., Rollence M. L. et al. (1989) Large increase in general stability for subtilisin BPN′ through incremental changes in the energy of unfolding. Biochemistry **28:** 7205–7213
104 Zhang X., Basse W. A., Shoichet B. K., Wilson K. P. and Matthews B. W. (1995) Enhancement of protein stability by the combination of point mutations in T4 lysozyme is additive. Protein Eng. **8:** 1017–1022
105 Ishikawa K., Nakamura H., Morikawa K., Kimura S. and Kanaya S. (1993) Cooperative stabilization of Escherichia coli ribonuclease HI by insertion of Gly-80b and Gly-77→Ala substitution. Biochemistry **32:** 7136–7142
106 Das G., Hickey D. R., McLendon D., McLendon G. and Sherman F. (1989) Dramatic thermostabilization of yeast iso-1-cytochrome c by an asparagine----isoleucine replacement at position 5. Proc. Natl. Acad. Sci. USA **86:** 496–499
107 Liao H., McKenzie T. and Hageman R., (1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. Proc. Natl. Acad. Sci. USA **83:** 576–580
108 Chen, K. and Arnold F. H. (1993) Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylfolmamide. Proc. Natl. Acad. Sci. USA **90:** 5618–5622
109 Kimura S., Nakamura H., Hashimoto T., Oobatake M. and Kanaya S. (1992) Stabilization of Escherichia coli ribonuclease HI by strategic replacement of amino acid residues with those from the thermophilic counterpart. J. Biol. Chem. **267:** 21535–21542
110 Vieille C. and Zeikus G. (1996) Thermozymes: identifying molecular determinants of protein structural and functional stability. TIB Tech. **14:** 183–190

111 Ito Y., Yamada H., Nakamura S. and Imoto T. (1990) Purification, amino acid sequence, and some properties of rabbit kidney lysozyme. J. Biochem. **107:** 236–241
112 Mitchison C. and Wells J. A. (1989) Protein engineering of disulfide bonds in subtilisin BPN′. Biochemistry **28:** 4807–4815
113 Imoto T., Yamada H. and Ueda T. (1986) Unfolding rates of globular proteins determined by kinetics of proteolysis. J. Mol. Biol. **190:** 647–649
114 Yamada H., Ueda T. and Imoto T. (1993) Thermodynamic and kinetic stabilities of hen-egg lysozyme and its chemically modified derivatives: analysis of the transition state of the protein unfolding. J. Biochem. **114:** 398–403
115 Fersht A. R., Matouschek A. and Serrano L. (1989) The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding. J. Mol. Biol. **224:** 771–782
116 Motoshima H., Ueda T. and Imoto T. (1996) Analysis of the transition state in the unfolding of hen lysozyme by introduction of Gly-Pro and Pro-Gly sequences at the same site. J. Biochem. **119:** 1019–1023
117 Mozhaev V. V. (1993) Mechanism-based strategies for protein thermostabilization. Trends Biotech. **11:** 88–59
118 Tomazic S. J. and Klibanov A. M. (1988) Why is one Bacillus alpha-amylase more resistant against irreversible thermoinactivation than another. J. Biol. Chem. **263:** 3092–3096
119 Tomizawa H., Yamada H. and Imoto T. (1994) The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 degrees C. Biochemistry **33:** 13032–13037
120 Tomizawa H., Yamada H., Wada K. and Imoto T. (1995) Stabilization of lysozyme against irreversible inactivation by suppression of chemical reactions. J. Biochem. **117:** 635–640
121 Tomizawa H., Yamada H., Tanigawa K. and Imoto T. (1995) Effects of additives on irreversible inactivation of lysozyme at neutral pH and 100 degrees C. J. Biochem. **117:** 369–373
122 Tomizawa H., Yamada H., Hashimoto Y. and Imoto T. (1995) Stabilization of lysozyme against irreversible inactivation by alterations of the Asp-Gly sequences. Protein Eng. **10:** 1023–1028
123 Imoto T., Ueda T., Tamura T., Isakari Y., Abe Y., Inoue M. et al. (1994) Lysozyme requires fluctuation of the active site for the manifestation of activity. Protein Eng. **7:** 743–748
124 Kraulis P. J. (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallogr. **24:** 946–950

.