Some Like It Hot: The Molecular Determinants of Protein Thermostability

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1. Introduction

Extremophilic organisms survive under harsh environmental conditions. They prefer to live at high hydrostatic pressure, in the presence of high salt concentrations, in acid or alkaline solutions, and, in particular, at high temperature.[1] The thermophiles and hyperthermophiles, some of which can grow beyond 110 °C, receive much attention, because their thermostable proteins have become important tools in biochemistry and industrial biotechnology. At the same time they provide us with the opportunity to elucidate the origins of protein stability, to learn how thermostability is encoded in the amino acid sequence, and, ultimately, to use this information for designing stable proteins.[2, 3]

Mesophilic organisms are not forced to maintain or evolve thermostable constituents and, as a consequence, most of their proteins show very low conformational stabilities. This may have a simple reason. The overwhelming majority of the mutations, as they occur during evolution, are disadvantageous for stability and function, and therefore the stability of a protein is maintained just high enough to secure its proper function in the organism.

Proteins are stabilized primarily by noncovalent interactions, such as hydrogen bonds, hydrophobic interactions, or coulombic forces. There is a multitude of interactions in proteins, but all of them are weak, they must balance the loss of interactions with the aqueous solvent and, in particular, they must compensate for the enormous decrease in chain entropy upon folding. Makhatadze and Privalov estimate a decrease in chain entropy equivalent to $T\Delta S \approx -1500\ \mathrm{kJ\,mol^{-1}}$ upon folding a protein with 100 residues.[4] It is thus not surprising that most proteins are only marginally stable, with Gibbs free energies of denaturation (ΔG_D) often in the range of 10 – 60 kJ mol^{-1}. The finely tuned balance between many stabilizing and many destabilizing interactions complicates the analysis, and it remains difficult to identify the molecular origins of the extra stability of the thermophilic proteins.

2. Stability and Rigidity of Thermophilic Proteins

At ambient temperature thermophilic proteins often show much slower amide hydrogen – deuterium exchange and much lower catalytic activity than their mesophilic homologues, which points to an increased conformational rigidity of the thermophilic proteins. In some cases these differences disappear when mesophilic and thermophilic proteins are compared at their respective physiological temperatures.[5] The underlying concept of "corresponding states" is sometimes meant to imply that there is a mechanistic linkage between low activity and high rigidity.[2, 3, 6] The often low catalytic activity at ambient temperature of thermophilic enzymes might, however, simply be a consequence of the fact that a thermophilic enzyme should not show a maximal activity, but an activity that is optimized for adequate physiological function at high temperature. This implies that the activity is fairly low at ambient temperature because catalysis by an enzyme, like all other chemical reactions, obeys the Arrhenius relationship. In addition, a low activity at ambient temperature might simply reflect that there is no evolutionary pressure on thermophilic organisms to maintain high metabolic activity in the cold. In fact, mesophilic enzymes can be made thermostable by protein engineering or directed evolution without compromising their high activity at low temperature.[7–10] This confirms that there is not necessarily a trade-off between enzyme stability and activity.

What makes a thermophilic protein stable? Usually, homologous proteins from mesophilic and thermophilic organisms strongly resemble each other in their three-dimensional structures. They use the same building blocks, the natural amino acids, but of course they differ in amino acid sequence. It is clear that the stability differences are determined by the differences in sequence, and therefore structure-based sequence comparison is the most popular approach to tackle the problem of protein thermostability.

However, the sequences of mesophilic and thermophilic protein homologues typically differ at many positions, and most of these differences are certainly unimportant for thermal adaptation. Therefore, it has remained a major challenge to pinpoint those differences that actually convey the additional stability to the thermophilic protein. Moreover, spatial information from crystal or NMR spectroscopic structures is not easily

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translated into thermodynamics. Interactions depend strongly on molecular distances and distance distributions. Entropic changes that accompany folding or the differential interactions of charged residues in the unfolded and native states cannot be deduced from the folded structure at all.[11]

Nevertheless, structure-aided sequence comparisons between mesophilic and thermophilic proteins provided initial insight into what might have guided the evolution of thermophilic proteins to increased stability. In a pioneering study, Argos et al. found tendencies for substitutions of, for example, Ser and Gly to Ala, Val to Ile, and Lys to Arg when going from the mesophilic to the thermophilic homologues.[12] Now, with several complete bacterial and archaeal genome sequences at hand it has become clear that thermophiles encode more charged and less uncharged and polar residues.[2, 13, 14] These global analyses are very useful but cannot reveal whether the observed trends (such as the preference in thermophiles of Glu and Asp over Gln and Asn) reflect the optimization of thermodynamic stability or of other factors, such as chemical stability at high temperature. They also cannot give us information about how a particular protein achieves its thermostability.

Many rules for protein thermostability were deduced from structure/sequence analyses, and, with the advent of site-directed mutagenesis, they could be tested by experiments. Most of these studies gave incomplete answers, because the often huge numbers of sequence differences rendered systematic residue-by-residue mutational analyses impossible. More importantly, most of the studied proteins unfolded irreversibly or followed complex unfolding mechanisms, both of which preclude a quantitative thermodynamic analysis of denaturation experiments. These limitations define the criteria for a mutational approach to protein thermostability. The mesophilic and thermophilic protein homologues under investigation should differ at a few sequence positions only, and they must unfold reversibly in a well-defined reaction, ideally in a simple N⇄U two-state process. For such reactions the equilibrium constant and, thus, the Gibbs free energy of denaturation, ΔG_D, can be calculated unambiguously and with good precision from unfolding transition curves.

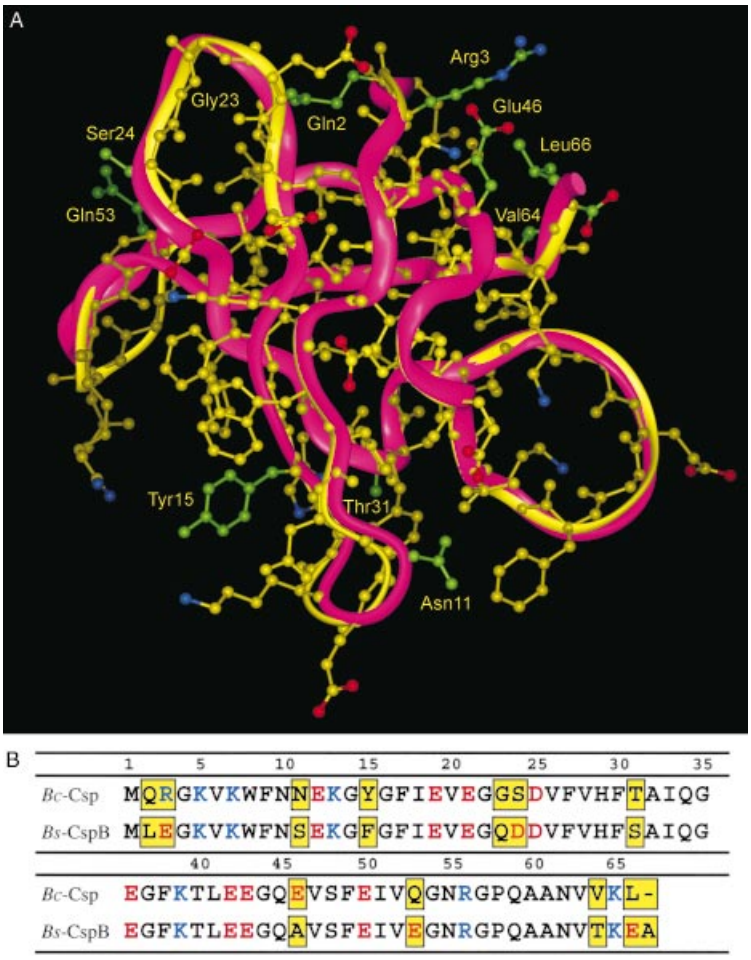

Figure 1. *A) Crystal structure of* Bc-*Csp.*[15] *The backbone is shown as a yellow ribbon, and the side chains that are identical between* Bc-*Csp and* Bs-*CspB are also shown in yellow. Those that are different from* Bs-*CspB are shown in green and labeled in yellow. Positively charged atoms are colored blue, negatively charged atoms are red. The backbone of* Bs-*CspB*[19] *is shown as a magenta ribbon. B) Amino acid sequences of* Bc-*Csp and* Bs-*CspB. The sequence differences are underlaid in yellow, negatively charged residues are shown in red, positively charged residues in blue. Single-letter notation for amino acids is used.*

3. Thermostability of the *Bacillus caldolyticus* Cold Shock Protein *Bc*-Csp

In our own studies on the origins of thermostability we used a mesophilic and a thermophilic cold shock protein as the workhorses.[15, 16] These small proteins bind to single-stranded nucleic acids, and, in the cells, they probably suppress secondary-structure formation in mRNAs by binding.[17, 18] The cold shock proteins from the mesophile *Bacillus subtilis* (*Bs*-CspB) and from the thermophile *Bacillus caldolyticus* (*Bc*-Csp; Figure 1) are monomeric, have 67 (*Bs*-CspB) and 66 (*Bc*-Csp) residues, and are devoid of complicating factors, such as disulfide bonds, coenzymes, or tightly-bound ligands. They differ in sequence at 12 positions only (Figure 1 B). The crystal structures, solved at resolutions of 2.45 (*Bs*-CspB) and 1.17 Å (*Bc*-Csp), revealed that the two proteins show the same backbone conformation (a β barrel composed of five antiparallel β strands) and share identical hydrophobic cores (Figure 1 A).[15, 19]

Both, *Bs*-CspB and *Bc*-Csp unfold and refold reversibly in very fast, monomolecular, N⇄U two-state reactions, there is no evidence for folding intermediates or for irreversible reactions. This pair of proteins is, thus, well suited for quantitative thermodynamic studies. The thermophilic protein is much more stable than its mesophilic homologue. In 5 mM buffer (pH 7.0) the midpoint of the thermal unfolding transition (T_M) of *Bc*-Csp is at 77.3 °C and is, thus, more than 28 degrees higher than the T_M of *Bs*-CspB. In 100 mM buffer the difference in T_M is 23 °C.[15, 16]

All twelve differences in sequence locate to the protein surface (Figure 1), and six of them involve charged groups. The resulting difference in surface-charge distribution is important for stability. This is clearly seen in the response of the stabilities of the two proteins to increasing the salt concentration in the solvent. The stability of the mesophilic protein *Bs*-CspB increases strongly when 0 – 0.5 M NaCl or KCl are added (Figure 2),

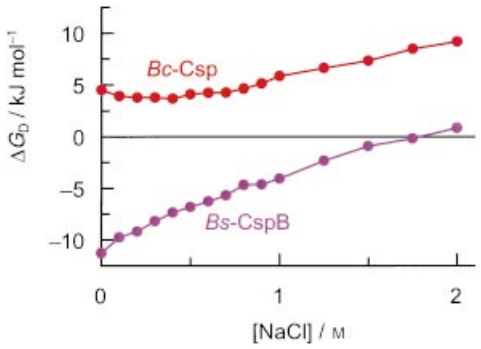

Figure 2. *Gibbs free energy of denaturation ΔG_D of wild-type* Bc-*Csp (red) and wild-type* Bs-*CspB (purple) at 70 °C for different NaCl concentrations as derived from thermal unfolding transitions. Transitions were measured in 0.1 M Na cacodylate/HCl (pH 7.0) at protein concentrations of 4 μM and monitored by the decrease of the circular dichroism signal at 222.6 nm. Data taken from ref. [16].*

evidently because unfavorable electrostatic interactions at the protein surface are screened by the salt. In contrast, the stability of the thermophilic protein *Bc*-Csp decreases under the same conditions, which suggests that in this case the electrostatic interactions are stabilizing. The stability difference between the two proteins, thus, decreases from 15.8 kJ mol^{-1} in 0.1 M buffer to 8.4 kJ mol^{-1} in 2 M NaCl solution; this suggests that the changes in the surface electrostatic interactions account for almost half of the additional stability of the thermophilic protein. At high salt concentrations, the stabilities of both proteins increase (Figure 2) in a charge-independent fashion due to the Hofmeister effect.[20–22]

4. Individual Contributions to Thermostability

Since the two cold shock proteins differ at twelve positions only, it was within reach to analyze the contributions of these differences by a combination of systematic mutagenesis and stability measurements. In a first series of mutations, we introduced into the thermophilic protein, one at a time, the corresponding residues of the mesophilic protein. The thermodynamic analysis of these twelve variants gave a surprisingly clear and simple answer. Only two of the twelve variants were significantly destabilized relative to wild-type *Bc*-Csp (see Figure 3 A), which suggests that the additional stability of this protein results from only the contributions of the Leu 66 and, in particular, Arg 3 residues. In *Bs*-CspB these positions are occupied by Glu residues. The Arg 3 Glu mutation alone destabilized *Bc*-Csp by 11.5 kJ mol^{-1}, which accounts for more than two thirds of its extra stability. When the two mutations were combined (in the Arg 3 Glu/Leu 66 Glu double mutant of *Bc*-Csp) the T_M value dropped to 44.6 °C, which is even below the T_M value of *Bs*-CspB (53.6 °C). The other amino acid replacements only led to small changes in stability (Figure 3 C). The $\Delta\Delta G_D$ increments of all the individual mutations added up to −15.1 kJ mol^{-1}, which is surprisingly close to the total difference in stability between the mesophilic and the thermophilic parent proteins (−15.8 kJ mol^{-1}).

It is always easy to destabilize a protein (such as *Bc*-Csp) by mutation. The salient question, therefore, was whether the mesophilic protein *Bs*-CspB could be stabilized by introducing the residues of the thermophilic homologue at the critical positions 3 and 66. The answer was clearcut. The Glu 3 Arg replacement increased the T_M value of *Bs*-CspB by 16 °C and ΔG_D by 11.1 kJ mol^{-1}, while the Glu 66 Leu mutation increased the T_M value by 12.8 °C and ΔG_D by 8.8 kJ mol^{-1} (Figure 3 B, D). In combination, the two mutations raised the T_M value from 53.6 to 74.6 °C and, thus, rendered *Bs*-CspB nearly as stable as *Bc*-Csp (76.9 °C).

The Arg 3 residue of *Bc*-Csp accounts not only for two thirds of the extra stability of this protein but also for the profound difference in the electrostatic stabilization between *Bs*-CspB and *Bc*-Csp. The exchange of a Glu residue at position 3 with an Arg residue was sufficient to switch the coulombic interactions from being overall destabilizing as in the mesophilic parent protein to being overall stabilizing as in the thermophilic parent protein (see Figure 2). All the other mutations (including the Leu/Glu exchange at position 66) did not affect the electrostatic interactions, although five of the substitutions involve changes in charge.

Can we understand the importance of the Arg 3 and Leu 66 residues for the thermostability of *Bc*-Csp on the basis of its

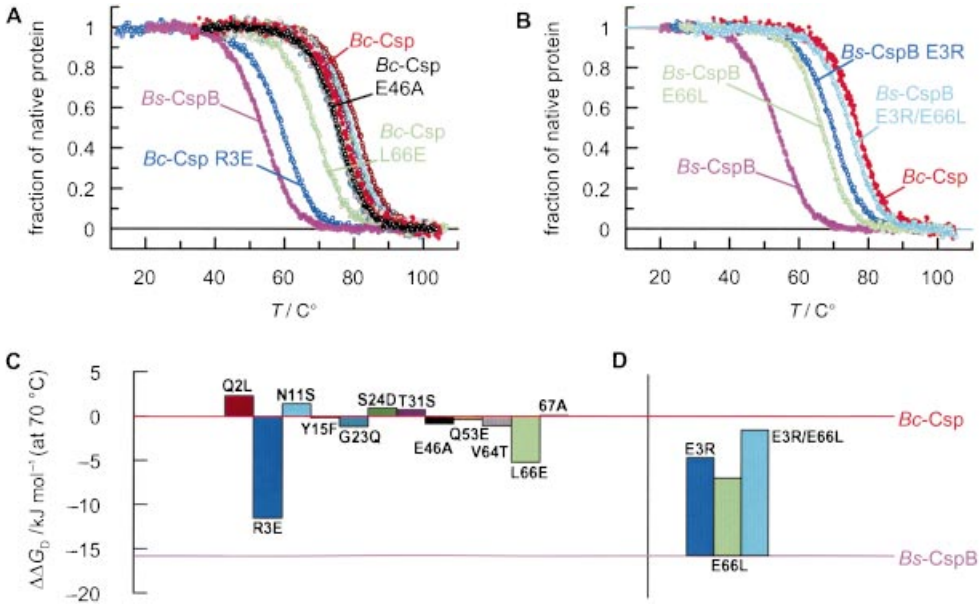

Figure 3. *Thermal unfolding transitions of A) the variants of* Bc-*Csp and B) the variants of* Bs-*Csp in 0.1 M Na cacodylate/HCl (pH 7.0). The conditions were as given in Figure 2. Single-letter notation for amino acids is used. C) The relative differences in stability ($\Delta\Delta G_D$) caused by the individual substitutions of the respective residues of* Bs-*CspB into* Bc-*Csp. D) The effects of selected reverse substitutions on the stability of Bs-CspB. The relative stabilities of the two parent proteins are indicated by the horizontal lines. Data taken from ref. [16].*

1.17 Å crystal structure? The side chain of the Leu 66 residue packs on a pair of main-chain hydrogen bonds between Val 47, Ser 48, and Val 64, which link two β strands. It thus decreases the polarity around these hydrogen bonds and excludes water molecules as potential hydrogen-bond competitors. This hydrophobic shielding might be the major source for the stabilization by the Leu 66 residue relative to a Glu 66 moiety. Interestingly, the Glu 66 residue is disordered in the structure of *Bs*-CspB.[19]

5. The Key Role of the Arg 3 Residue

The Arg 3 residue stabilizes both wild-type *Bc*-Csp and the Glu 3 Arg variant of *Bs*-CspB by more than 11 kJ mol^{-1} relative to the forms that have a Glu residue at this position. Of this, 7 kJ mol^{-1} are of coulombic origin, because screening by 2 M salt concentrations reduces ΔG_D to 4.2 kJ mol^{-1}. This salt-resistant stabilization might reflect improved nonpolar interactions of the three methylene groups of the Arg 3 side chain.

The Arg 3 residue shows two different side-chain conformations in the two protein molecules (A and B) that are found in the unit cell of the *Bc*-Csp crystals. The distances of its guanidinyl group to the carboxyl group of the Glu 46 residue are 4.8 Å in molecule A and 2.7 Å in molecule B,[15] which raises the possibility that the Arg 3 and Glu 46 residues engage in a stabilizing salt bridge. The control mutation Glu 46 Ala, however, left the stability of *Bc*-Csp and its salt dependence virtually unchanged, which rules out the idea that the strong electrostatic stabilization by the Arg 3 moiety originates, in fact, from a pairwise ionic interaction with the Glu 46 residue. We then abolished, one at a time, all other negatively charged groups that are closer than 10 Å to the Arg 3 residue, by mutagenesis, analyzed the stabilities of the respective variants by double-mutant cycles (D. Perl, unpublished results), and found that none of them serves as a salt-bridge partner for the Arg 3 residue.

At least part of the electrostatic stabilization that accompanies the replacement of the Glu 3 residue with Arg might originate from removing ionic repulsions with Glu residues at position 46 and/or position 66. The Cβ atoms of residues 3, 46, and 66 (Figure 4 A) are closer than 8 Å in the crystal structures of the two cold shock proteins. To analyze the differential energetic interactions that originate from the residues at positions 3, 46, and 66 as present in the thermophilic (Arg 3, Glu 46, Leu 66) and the mesophilic proteins (Glu 3, Ala 46, Glu 66) we combined them in all possible ways, which leads to eight variants that can be arranged as a triple-mutant cube, as shown in Figure 4 B. The thermodynamic analyses of all species of this cube gave a surprisingly simple answer. Whenever Glu residues are present at positions 3 and 46, or at positions 3 and 66, they repel each other electrostatically and destabilize the protein by 4 kJ mol^{-1}, independent of the nature of the third residue (D. Perl, unpublished results). These repulsion energies vanished in the presence of 2 M NaCl solution, as expected for ionic interactions that can be screened by counterions. These results explain why the destabilization by the Glu 3 relative to the Arg 3 residue in *Bc*-Csp is as strong as the stabilization by the reverse mutation in *Bs*-CspB. In both cases the Glu 3 residue is involved in a repulsion, in

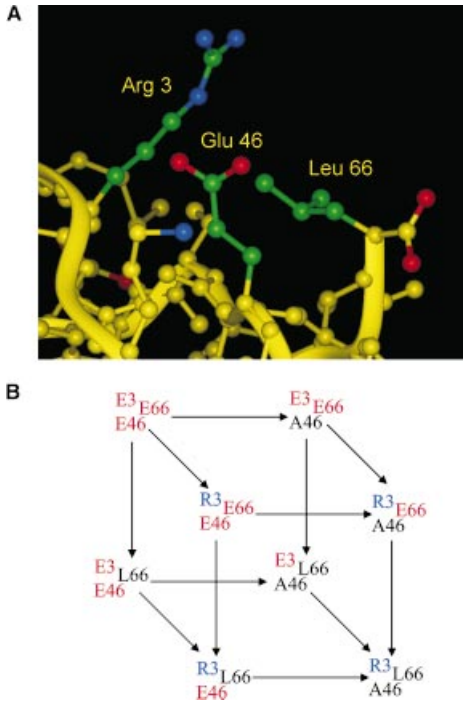

Figure 4. *A) Close-up view of the Arg 3, Glu 46, and Leu 66 residues in the crystal structure of* Bc-*Csp.*[15] *The backbone is shown as a yellow ribbon, the side chains at positions 3, 46, and 66 are shown in green, the other side chains are in yellow. Positively charged atoms are colored blue, negatively charged atoms are red. B) Triple-mutant cycle for* Bc-*Csp. The Arg 3, Glu 46, and Leu 66 residues were substituted by the respective amino acids as they occur in* Bs-*CspB (Glu 3, Ala 46, Glu 66) in all possible combinations to determine coupling energies. Single-letter notation for amino acids is used. Negatively charged residues are notated in red, positively charged residues in blue, and uncharged residues in black. The six double-mutant cycles that make up the faces of the cube are analyzed as if they represented thermodynamic cycles. Mutant cycle analyses are described in ref. [57].*

Bs-CspB with the Glu 66 moiety and in the Arg 3 Glu mutant of *Bc*-Csp with the Glu 46 moiety. It also explains why the Arg 3 Glu/Leu 66 Glu double mutant of *Bc*-Csp is 4 kJ mol^{-1} less stable than expected from the sum of the effects of the single mutants. In this double mutant, positions 3, 46, and 66 are all occupied by Glu residues, which leads to two repulsions (whereas *Bs*-CspB has Glu residues only at positions 3 and 66).

In summary, the Arg 3 residue stabilizes *Bc*-Csp by three mechanisms. 1) The aliphatic moiety of the Arg 3 residue makes better nonpolar interactions than Glu and contributes 4.2 kJ mol^{-1} of stabilization energy in a salt-independent fashion. 2) The relief of the coulombic repulsion between the Glu 3 and Glu 46 residues contributes 4.2 kJ mol^{-1}. 3) The remaining 3 kJ mol^{-1} originate from a general coulombic stabilization by the positively charged arginine side chain, which does not involve pairwise charge interactions. The contributions (2) and (3) vanish in the presence of 2 M NaCl solution.

The repulsive interactions between Glu residues at positions 3 and 66 or 46 are not replaced by a corresponding attractive interaction when the Glu 3 residue is changed to Arg, as we had guessed initially. In retrospect, this is not surprising. When two like charges approach each other, repulsion leads to an unfavorable (positive) enthalpy contribution and restricts the

conformational mobility, that is, the change in entropy is also unfavorable. Both effects add up to a strong destabilization. When the two residues are oppositely charged, however, electrostatic attraction lowers the enthalpy, which is favorable, but the corresponding restriction in mobility also lowers the entropy, which is unfavorable. Apparently, in our case, the gain in enthalpy was not high enough to pay for the loss in conformational mobility and, therefore, the salt bridge did not form. Indeed, the side chain of the Arg 3 residue showed conformational heterogeneity in the high-resolution crystal structures of *Bc*-Csp and several mutants,[15] which points to the high conformational entropy of its side chain. This may explain why attempts to graft allegedly stabilizing salt bridges from thermophilic to mesophilic proteins have failed in many cases.[23, 24]

Interestingly, the two crucial positions for the additional thermostability of *Bc*-Csp (Arg 3 and Leu 66) are close to the chain termini. Kirschner and others had proposed earlier that fixation of the chain ends should be an efficient strategy for making proteins thermostable.[25, 26]

Systematic mutational analyses of the origins of protein thermostability were also performed for two histone-like proteins, the HU protein from the thermophile *Bacillus stearothermophilus* and the H*Mf*B protein from the hyperthermophile *Methanothermus fervidus*.[27, 28] Both proteins are dimeric, and they are stabilized predominantly by the strengthening of the intermolecular interactions between the subunits, but also by improved ionic interactions within the subunits.

6. The Role of Surface-Exposed Charges for Protein Thermostability

There is a broad agreement between experimentalists and theoreticians that surface-exposed charged residues can be important for the stability of proteins in general and of thermophilic proteins in particular.[11, 26, 29–34] It has remained unclear, however, whether this stabilization involves specific pairwise electrostatic interactions, as suggested by the analyses of the three-dimensional structures of thermophilic proteins.[2, 26, 28] The distances between oppositely charged residues can easily be measured in protein structures, and, in simplistic approaches, distances shorter than a certain cut-off (for example, 4 – 6 Å) are translated into stabilizing ion pairs. It is now clear that such a conversion of distances into energies can be misleading. This is exemplified by the analysis of the putative salt bridge between the Glu 14 residue and the amino terminus of a hyperthermophilic rubredoxin. A double-mutant cycle uncovered a stabilizing interaction between the two charged groups, but this interaction was more stable in the ionic denaturant GdmCl than in urea, which does not screen ionic interactions.[35] Other mutational analyses of putative salt bridges have given ambiguous answers.[27, 36–42]

The formation of pairwise salt bridges is opposed not only by the unfavorable loss in side-chain entropy but also by the desolvation of the interacting charged groups. Charges are easier to desolvate at high temperature because the liberated water molecules gain more in $T\Delta S$, and, therefore, it was suggested that salt bridges are, in fact, more stable in thermophilic proteins.[29, 31]

Alternatively, extended arrays of surface charges in thermophilic proteins have been proposed to enhance stability. In such networks the entropic penalty would be much smaller than for pairwise interactions, because the charged groups of the network can engage in multiple interactions. It has, however, remained difficult to elucidate the energetics of such complex networks by directed mutagenesis and to transplant them to mesophilic proteins.[23, 24]

All these difficulties of localizing electrostatic effects to pairwise interactions have revived the interest in the overall electrostatic properties of proteins. Ladenstein, Karshikoff, and co-workers analyzed a large set of protein structures and concluded that thermophilic proteins are better optimized electrostatically,[33, 43] and, in particular, that repulsive contacts are reduced, which is in very good agreement with our study of the two cold shock proteins. Xiao and Honig also argue that structure/sequence comparisons and the counting of putative salt bridges provide little insight into the origins of thermostability.[32] Rather, they suggest that, in thermophilic proteins, the charges are optimally placed to improve the overall electrostatic interactions.

Electrostatic calculations on proteins have a long history, all the way back to the classical work of Tanford and Kirkwood.[44] Recently, several groups used calculations based on Coulomb's law to analyze electrostatic interactions, and, by this approach, surface point mutations could be identified that stabilized ubiquitin,[45] the ribonucleases T1 and Sa, and a fragment of the pyruvate dehydrogenase multienzyme complex.[46] Calculations to increase the stability of the cold shock protein electrostatically have also been performed (C. Brooks III, personal communication). These strategies are very promising for detecting unfavorable electrostatic interactions.

7. How to Stabilize Proteins

Robust proteins are of great interest for biotechnology. Initial attempts to stabilize proteins by site-directed mutagenesis were often unsuccessful, and they revealed that we knew much less about the principles of protein stability than we thought in the days when planned sequence manipulations were still out of reach. Methods of directed evolution can be used to make large libraries of protein variants by random mutagenesis and to search them for stabilized ones by a screening or selection step. These approaches suffer from the limited sequence space that is available from single nucleotide variations as well as from the fact that single amino acid exchanges are mostly destabilizing, in particular when they occur in the protein interior.[3]

Several proteins have been strongly stabilized by combining data from sequence comparisons and from rationally designed mutations. By such an approach, the thermolysin-like protease from *Bacillus stearothermophilus* could be engineered to resist boiling through eight amino acid substitutions.[8] Cytochrome c_{551} from the mesophile *Pseudomonas aeruginosa* became nearly as stable as its thermophilic counterpart from *Hydrogenobacter*

thermophilus by changing only five amino acids, although the two proteins share only 56% sequence identity.[47]

The combination of our results with previous analyses points to new avenues for stabilizing proteins. We propose a strategy in which surface sites are first identified that carry a potential for stabilization. Exposed sites are very attractive candidates, because, unlike core positions, they can accommodate many different residues, show few intraprotein interactions, and, thus, tend to contribute to stability in an additive fashion. Suitable positions could be identified by stability algorithms or by electrostatics calculations.[32, 48–51] Repulsive interactions, in particular, should be easy to identify. The most promising sites can then be randomized by saturation triplet mutagenesis, and the resulting libraries of variants be searched for variants with improved stability by a screening or selection procedure. Several selection procedures that are based on phage- or ribosome-display systems are available to select stabilized variants out of large libraries.[52–56]

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[1] R. Huber, H. Huber, K. O. Stetter, *FEMS Microbiol. Rev.* **2000**, *24*, 615 – 623.
[2] R. Jaenicke, G. Böhm, *Curr. Opin. Struct. Biol.* **1998**, *8*, 738 – 748.
[3] P. L. Wintrode, F. H. Arnold, *Adv. Protein Chem.* **2000**, *55*, 161 – 225.
[4] G. I. Makhatadze, P. L. Privalov, *Adv. Protein Chem.* **1995**, *47*, 307 – 425.
[5] P. Zavodszky, J. Kardos, Svingor, G. A. Petsko, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7406 – 7411.
[6] G. N. Somero, *Annu. Rev. Physiol.* **1995**, *57*, 43 – 68.
[7] L. Giver, A. Gershenson, P. O. Freskgard, F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12809 – 12813.
[8] B. Van den Burg, G. Vriend, O. R. Veltman, G. Venema, V. G. Eijsink, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2056 – 2060.
[9] K. Miyazaki, P. L. Wintrode, R. A. Grayling, D. N. Rubingh, F. H. Arnold, *J. Mol. Biol.* **2000**, *297*, 1015 – 1026.
[10] R. Sterner, W. Liebl, *Crit. Rev. Biochem. Mol. Biol.* **2001**, *36*, 39 – 106.
[11] C. N. Pace, R. W. Alston, K. L. Shaw, *Protein Sci.* **2000**, *9*, 1395 – 1398.
[12] P. Argos, M. G. Rossmann, U. Grau, H. Zuber, G. Frank, J. Tratschin, *Biochemistry* **1979**, *18*, 5698 – 5703.
[13] P. J. Haney, J. H. Badger, G. L. Buldak, C. I. Reich, C. R. Woese, G. J. Olsen, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3578 – 3583.
[14] C. Cambillau, J. M. Claverie, *J. Biol. Chem.* **2000**, *275*, 32383 – 32386.
[15] U. Mueller, D. Perl, F. X. Schmid, U. Heinemann, *J. Mol. Biol.* **2000**, *297*, 975 – 988.
[16] D. Perl, U. Mueller, U. Heinemann, F. X. Schmid, *Nat. Struct. Biol.* **2000**, *7*, 380 – 383.
[17] P. L. Graumann, M. A. Marahiel, *Trends Biochem. Sci.* **1998**, *23*, 286 – 290.
[18] A. Brandi, R. Spurio, C. O. Gualerzi, C. L. Pon, *EMBO J.* **1999**, *18*, 1653 – 1659.
[19] H. Schindelin, M. A. Marahiel, U. Heinemann, *Nature* **1993**, *364*, 164 – 168.
[20] P. H. von Hippel, K.-Y. Wong, *J. Biol. Chem.* **1965**, *240*, 3909 – 3923.
[21] R. L. Baldwin, *Biophys. J.* **1996**, *71*, 2056 – 2063.
[22] M. T. Record, W. Zhang, C. F. Anderson, *Adv. Protein Chem.* **1998**, *51*, 281 – 353.
[23] J. H. Lebbink, S. Knapp, J. van der Oost, D. Rice, R. Ladenstein, W. M. de Vos, *J. Mol. Biol.* **1998**, *280*, 287 – 296.
[24] J. H. Lebbink, S. Knapp, J. van der Oost, D. Rice, R. Ladenstein, W. M. de Vos, *J. Mol. Biol.* **1999**, *289*, 357 – 369.
[25] A. Merz, M. C. Yee, H. Szadkowski, G. Pappenberger, A. Crameri, W. P. C. Stemmer, C. Yanofsky, K. Kirschner, *Biochemistry* **2000**, *39*, 880 – 889.
[26] M. Hennig, B. Darimont, R. Sterner, K. Kirschner, J. N. Jansonius, *Structure (London)* **1995**, *3*, 1295 – 1306.
[27] W. T. Li, J. W. Shriver, J. N. Reeve, *J. Bacteriol.* **2000**, *182*, 812 – 817.
[28] S. Kawamura, Y. Abe, T. Ueda, K. Masumoto, T. Imoto, N. Yamaski, M. Kimura, *J. Biol. Chem.* **1998**, *273*, 19982 – 19987.
[29] I. Korndörfer, B. Steipe, R. Huber, A. Tomschy, R. Jaenicke, *J. Mol. Biol.* **1995**, *246*, 511 – 521.
[30] A. H. Elcock, *J. Mol. Biol.* **1998**, *284*, 489 – 502.
[31] P. I. de Bakker, P. H. Hunenberger, J. A. McCammon, *J. Mol. Biol.* **1999**, *285*, 1811 – 1830.
[32] L. Xiao, B. Honig, *J. Mol. Biol.* **1999**, *289*, 1435 – 1444.
[33] A. Karshikoff, R. Ladenstein, *Protein Eng.* **1998**, *11*, 867 – 872.
[34] S. Kumar, B. Ma, C. J. Tsai, R. Nussinov, *Proteins: Struct. Funct. Genet.* **2000**, *38*, 368 – 383.
[35] P. Strop, S. L. Mayo, *Biochemistry* **2000**, *39*, 1251 – 1255.
[36] B. W. Matthews, *Curr. Opin. Struct. Biol.* **1991**, *1*, 17 – 21.
[37] A. K. Meeker, B. Garcia-Moreno, D. Shortle, *Biochemistry* **1996**, *35*, 6443 – 6449.
[38] D. Sali, M. Bycroft, A. R. Fersht, *J. Mol. Biol.* **1991**, *220*, 779 – 788.
[39] L. Serrano, A. Horovitz, B. Avron, M. Bycroft, A. R. Fersht, *Biochemistry* **1990**, *29*, 9343 – 9352.
[40] K. Takano, K. Tsuchimori, Y. Yamagata, K. Yutani, *Biochemistry* **2000**, *39*, 12375 – 12381.
[41] N. Frankenberg, C. Welker, R. Jaenicke, *FEBS Lett.* **1999**, *454*, 299 – 302.
[42] D. N. Marti, I. Jelesarov, H. R. Bosshard, *Biochemistry* **2000**, *39*, 12804 – 12818.
[43] V. Z. Spassov, A. D. Karshikoff, R. Ladenstein, *Protein Sci.* **1994**, *3*, 1556 – 1569.
[44] C. Tanford, J. G. Kirkwood, *J. Am. Chem. Soc.* **1957**, *79*, 5333 – 5339.
[45] V. V. Loladze, B. Ibarra-Molero, J. M. Sanchez-Ruiz, G. I. Makhatadze, *Biochemistry* **1999**, *38*, 16419 – 16423.
[46] S. Spector, M. Wang, S. A. Carp, J. Robblee, Z. S. Hendsch, R. Fairman, B. Tidor, D. P. Raleigh, *Biochemistry* **2000**, *39*, 872 – 879.
[47] J. Hasegawa, S. Uchiyama, Y. Tanimoto, M. Mizutani, Y. Kobayashi, Y. Sambongi, Y. Igarashi, *J. Biol. Chem.* **2000**, *275*, 37824 – 37828.
[48] M. Akke, S. Forsen, *Proteins: Struct. Funct. Genet.* **1990**, *8*, 23 – 29.
[49] B. Ibarra-Molero, V. V. Loladze, G. I. Makhatadze, J. M. Sanchez-Ruiz, *Biochemistry* **1999**, *38*, 8138 – 8149.
[50] G. R. Grimsley, K. L. Shaw, L. R. Fee, R. W. Alston, B. M. Huyghues-Despointes, R. L. Thurlkill, J. M. Scholtz, C. N. Pace, *Protein Sci.* **1999**, *8*, 1843 – 1849.
[51] J. J. Havranek, P. B. Harbury, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11145 – 11150.
[52] A. Martin, V. Sieber, F. X. Schmid, *J. Mol. Biol.* **2001**, *309*, 717 – 726.
[53] V. Sieber, A. Plückthun, F. X. Schmid, *Nat. Biotechnol.* **1998**, *16*, 955 – 960.
[54] P. Kristensen, G. Winter, *Folding Des.* **1998**, *3*, 321 – 328.
[55] M. D. Finucane, M. Tuna, J. H. Lees, D. N. Woolfson, *Biochemistry* **1999**, *38*, 11604 – 11612.
[56] J. Hanes, L. Jermutus, S. Weber-Bornhauser, H. R. Bosshard, A. Plückthun, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 14130 – 14135.
[57] A. Horovitz, *Folding Des.* **1996**, *1*, R121-R126.

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