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The Contribution of Cross-Links to Protein Stability: A Normal Mode Analysis of the Configurational Entropy of the Native State

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ABSTRACT **The vibrational entropy of native BPTI, with three disulfide bonds, was determined by use of normal mode calculations and compared with that of folded variants having either one less disulfide bond or lacking a peptide bond at the trypsin-reactive site. Favorable contributions to the free energy of 2.5–5.1 kcal/mol at 300 K were calculated for the reduction of disulfide bonds in the folded state, whereas no favorable contribution was found for the hydrolysis of the peptide bond cleaved by trypsin. This is on the order of the effect of disulfides in the unfolded state. The implications of these results for the stabilization of a folded protein by the introduction of cross-links are discussed.** © 1993 Wiley-Liss, Inc.

Key words: disulfide bonds, protein stability, entropy of proteins

INTRODUCTION

Intramolecular cross-links can be used to stabilize proteins. Upon folding into a globular structure, a polypeptide chain loses considerable conformational freedom.[1] This restriction of the backbone and side chains contributes unfavorably to the entropy of folding. Over 30 years ago, it was suggested that the introduction of covalent cross-links could decrease this effect.[2–5] A cross-link whose only consequence is to significantly decrease the conformational space available to the unfolded chain should make the entropy of chain folding less unfavorable; that is, by decreasing the chain entropy of the unfolded state, the loss in going to the folded state is reduced. If there is only one disulfide bond, the expected effect increases with the distance between the cross-linked partners in the primary sequence;[3] if there are several disulfides, the behavior is more complex.[4,6,7]

Cross-links can be introduced in several ways. By use of site-specific mutagenesis, it is now possible to introduce either a second cysteine near an existing one or two new cysteines where none existed before. Conditions can then be chosen to create a disulfide linkage. This has been done in a number of proteins, including subtilisin,[8,9] T4 lysozyme,[10–12] dihydrofolate reductase,[13,14] and λ repressor.[15] Using chemical modification, cross-links have also been introduced into ribonuclease A,[16] hen egg-white lysozyme,[17] and bovine pancreatic trypsin inhibitor.[18,19] Interestingly, the results have not always increased the stability of the native state. To determine the effect of any change (e.g., amino acid sequence, cross-link, or chemical structure) on protein stability, one must consider both the unfolded and the folded state.[20–22] Indeed, investigation of cross-links engineered into subtilisin that did not increase the stability of the enzyme indicated that the covalent modifications introduced strain in the folded structure (both in "high-energy" dihedral angles and in close contacts) which offset the expected gain in chain entropy.[23] Methods have been developed to determine locations in proteins where relatively unstrained disulfide bonds can be introduced.[24–27]

Static analyses of structural strain neglect an additional destabilizing contribution of cross-links to the stability of the folded state. As was pointed out by Johnson et al. some years ago[28] and more recently by Goldenberg,[29] a cross-link can decrease the conformational freedom of the folded state as well as that of the unfolded state. Put another way, just as a cross-link can introduce "enthalpic strain" in the folded state, it can also introduce "entropic strain" in that state. The folded protein is a fluctuating structure undergoing significant internal motion.[30] Consequently, there is a certain residual configurational entropy associated with the folded state that is likely to decrease upon the addition of a cross-link.[31]

To study this effect, we chose the bovine pancreatic trypsin inhibitor (BPTI). This globular protein has 58 residues, and the wild-type molecule has 6 cysteines involved in three disulfide bonds (14–38, 30–51, and 5–55). Rather than introducing additional covalent cross-links, we examined the effect of removing them. In particular, we studied each of the

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three molecules with one reduced disulfide bond and the molecule with the peptide bond between Lys-15 and Ala-16 hydrolyzed (the putative product of cleavage by trypsin). The entropies of these four molecules in their folded state were compared with that of the native form with three disulfide bonds. The choice of this system for study was based on the existence of structural data indicating that the two-disulfide species have structures that are very similar to the native form[32-36] and activity measurements showing that the hydrolyzed form is also a potent inhibitor of trypsin.[37] Moreover, thermodynamic measurements of the effect of reducing the disulfide bonds on the stability have been reported.[29,32,38] Studies have also been reported on the stabilities of derivatives of ribonuclease T_1 with one and both of the native disulfide bonds removed.[39]

The internal motions of the five systems were modeled with harmonic dynamics. Harmonic models are often used in the study of proteins[40-42] and nucleic acids.[43-46] They are particularly useful because, although the model uses a harmonic approximation to the full potential employed in molecular dynamics simulations, the solution to the equations of motion can be determined exactly. Moreover, the solution is identical to that of a large number of independent harmonic oscillators. The harmonic oscillator model (one of the most thoroughly studied systems in physics) has simple equations that may be used to find the fluctuations and correlations of internal motions as well as the quantum mechanical partition function needed for the evaluation of thermodynamic quantities,[47,48] such as the free energy, the enthalpy, the entropy, and the heat capacity. Recently harmonic models of B- and Z-DNA have been useful in calculating the entropy difference between these two forms of a simple hexamer,[44] and an all-hydrogen harmonic model for BPTI has been employed in an analysis of data from inelastic neutron scattering experiments.[49] Since the neutron scattering results are sensitive to the density of states (number of vibrational modes per frequency interval), the observed agreement indicates that harmonic models can yield accurate densities of states. This is of particular importance for the vibrational entropy, since it depends only on the frequencies and their distribution.

The results of the calculations show that cross-links can, indeed, reduce the internal entropy of the folded molecule. This reflects the smaller fluctuations of the atoms due to the presence of cross-links. However, if a covalent cross-link is introduced where a strong noncovalent interaction existed before, there may be no entropy reduction.

An alternative approach would be to use a free energy simulation method[22] to calculate the free energy change for reducing each disulfide bond in the folded state. Although the three values would have no absolute meaning, they could be compared with each other or with an appropriate reference system (e.g., reducing the disulfide in solvated glutathione or DTT). Such simulations are very useful for calculating free energy changes and for decomposing the results into contributions from particular favorable and unfavorable interactions.[22] However, they have so far been less successful in determining entropies and enthalpies due to the slower convergence of these thermodynamic quantities. There is also a question of whether standard free energy simulations, which focus on local changes, would be applicable to the possibly more global effects involved in the entropy change being considered here. Since our goal was to study the effect of cross-links on the internal entropy, we chose a harmonic analysis rather than a free energy simulation.

We first summarize the model used and the method employed for the calculations. We then present the results and discuss their implications. Finally, we outline the conclusions.

MODEL AND METHOD

All molecular mechanics calculations were carried out with the computer program CHARMM[50] on a Cyber 205; the Cyber 205 version was prepared by one of the authors (BT). BPTI was modeled using all nonhydrogen atoms for the protein plus hydrogens that are bonded to oxygen and nitrogen atoms (the polar-hydrogen representation). All other hydrogens were treated as part of the carbon or sulfur atom to which they are bonded. The CHARMM-adapted TIP3P model of water was used for the four internal solvent molecules. The general methods for calculating the energy and its first two derviatives have been described elsewhere.[41,42,50]

The results of normal mode calculations depend on the manner in which the long-range electrostatic energy term is treated at large distances. Calculations for each structure were done with a model similar to that which gives good agreement with the neutron scattering data.[49] Electrostatic and van der Waals interactions were switched smoothly to zero with a cubic function between 6.5 and 7.5 Å. (This is the atom based electrostatic and van der Waals SWITCH cutoff in the CHARMM program.[50]) All interactions beyond 7.5 Å did not contribute to the energy and its derivatives.

Starting from the X-ray crystal structure for the non-hydrogen atoms of BPTI and the four internal solvent molecules,[51] the hydrogen positions were generated using HBUILD.[52] Five structures were considered: (1) native BPTI, (2) BPTI with disulfide 14–38 reduced, denoted [30–51, 5–55], (3) BPTI with disulfide 30–51 reduced, [14–38, 5–55], (4) BPTI with disulfide 5–55 reduced, [14–38, 30–51], and (5) BPTI with the peptide bond between Lys-15 and Ala-16 hydrolyzed. In each reduced species the disulfide bond was replaced by two SH groups in the crystal structure and then the structure was energy

TABLE I. Results of Energy Minimization

Model	Rms derivative [$\times 10^{-6}$ kcal/(mol-Å)]	Rms difference (Å) from Crystal	Rms difference (Å) from Native model
1. Native	0.7	1.011	—
2. [30–51, 5–55]	2.6	1.033	0.418
3. [14–38, 5–55]	1.1	0.961	0.444
4. [14–38, 30–51]	0.6	0.982	0.607
5. Hydrolyzed	2.0	1.095	0.778

minimized. The last model has all three disulfide bonds intact, but the peptide bond has been hydrolyzed to give a carboxylate group at Lys-15 and a protonated amino group at Ala-16. This is the expected result of the cleavage of this bond by trypsin.

The normal mode method expands the energy as a Taylor series about a local minimum. Each model was energy minimized to a root-mean-squared (rms) energy derivative of less than 5×10^{-6} kcal/(mol-Å). Energy minimization began with 2,500 steps of the steepest descent algorithm (to relax the structure in the neighborhood of the nearest energy minimum) and continued with the adopted-basis Newton–Raphson (ABNR) algorithm[30,50] for 7,500 steps. The nonbonded interaction list was updated every 50 steps during these minimizations. The appropriately mass-weighted second-derivative matrix was calculated[42,53] and the complete set of eigenvalues and eigenvectors was determined using MAGEV (subroutines TRED2 and IMTQL).[54] MAGEV is a mathematics package that has been highly optimized for the Cyber 205. Matrix diagonalization (complete eigenvalue and eigenvector extraction) for each real, symmetric matrix [1,740 × 1,740 for structures (1)–(4); 1,749 × 1,749 for structure (5)] required under 11 CPU minutes on a Cyber 205 with four million words of memory and two vector pipelines. In each case, there were six zero-frequency modes (actual values were less than 7.2×10^{-3} cm^{-1} in magnitude in all cases) corresponding to overall translation and rotation of the system. There were no negative eigenvalues. Thermodynamic properties for each system were calculated quantum mechanically using the frequencies of the internal (nontranslation/rotation) modes for each system from,[47,48]

$$-TS(vib) = \sum_{i=1}^{3N-6} \left[\frac{-h\nu_i}{e^{\frac{h\nu_i}{k_BT}} - 1} + k_BT \ln\left(1 - e^{\frac{-h\nu_i}{k_BT}}\right) \right] \quad (1)$$

and

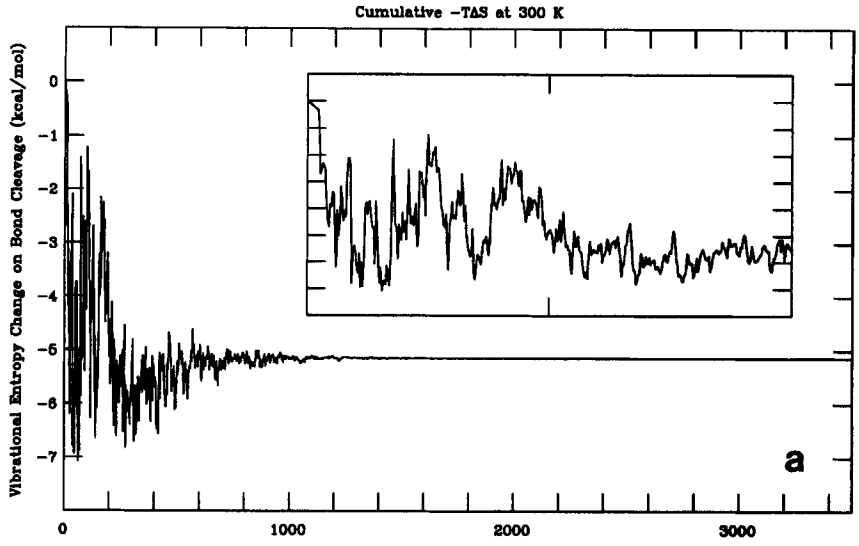
$$\langle \Delta r_i^2 \rangle = \frac{k_BT}{m_i} \sum_{j=1}^{3N-6} \frac{a_{ij}^2}{\nu_j^2} \quad (2)$$

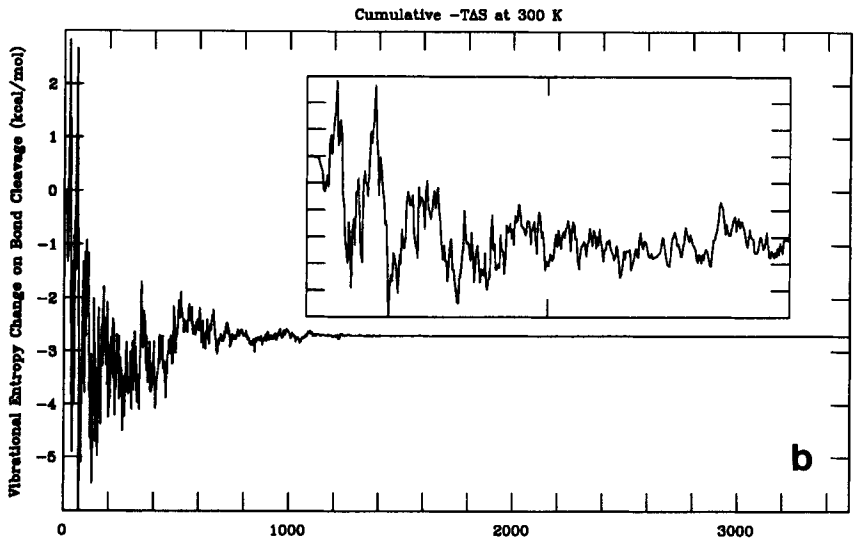
where k_B is the Boltzmann constant, T is the absolute temperature, N is the number of atoms (giving $3N$—6 vibrational modes), h is the Planck constant, ν_i is the frequency of the ith normal mode, $\langle \Delta r_i^2 \rangle$ is the mean-squared fluctuation of the ith coordinate, m_i is its associated mass, and a_{ij} is the ith coordinate of the jth normal mode for an orthonormalized set.

RESULTS AND DISCUSSION

Each system minimized to a structure close to the native crystallographic structure. Table I lists the rms differences between crystal and minimized coordinate sets as well as the final gradients. Each structure moved by about 1 Å rms from the crystal structure and the maximum rms gradient for the five minimized structures is 2.6×10^{-6} kcal/(mol-Å). Experimental results on the reduced-disulfide molecules suggest that the structure with disulfide 14–38 reduced is most similar to native, the reduction of 30–51 causes the next smallest structural perturbation, and the reduction of 5–55 causes the largest change in structure.[32,33] Comparison of the minimized disulfide bond-broken structures with the minimized native structure suggests that the calculated shifts agree with the qualitative experimental results. The X-ray structure of the double mutant (Cys 30 → Ala and Cys 51 → Ala), in which one disulfide is removed and atomic volume is lost from the hydrophobic core, shows some small shifts relative to native that reduce the packing defect caused by the mutations.[35] In the work presented here, the two-disulfide species were studied in which the third disulfide was reduced to a pair of sulfhydryls. Since this does not result in a decrease in atomic volume (in fact, it causes a slight increase), the shifts that relieve cavities introduced in the double alanine mutant are not seen here. There are no structural data on the hydrolyzed bond structure, though the fact that it binds strongly to trypsin suggests that the structure is similar to the native one. Each structure represents a local energy minimum close to the crystal structure; the actual structures for some of these molecules in solution may involve larger distortions than energy minimization alone can produce.

The entropic contributions to the free energy are shown in Table II. Reduction of any of the three


Cumulative -TΔS at 300 K
Vibrational Entropy Change on Bond Cleavage (kcal/mol)
0
-1
-2
-3
-4
-5
-6
-7
0
1000
2000
3000
a


Cumulative -TΔS at 300 K
Vibrational Entropy Change on Bond Cleavage (kcal/mol)
2
1
0
-1
-2
-3
-4
-5
0
1000
2000
3000
b

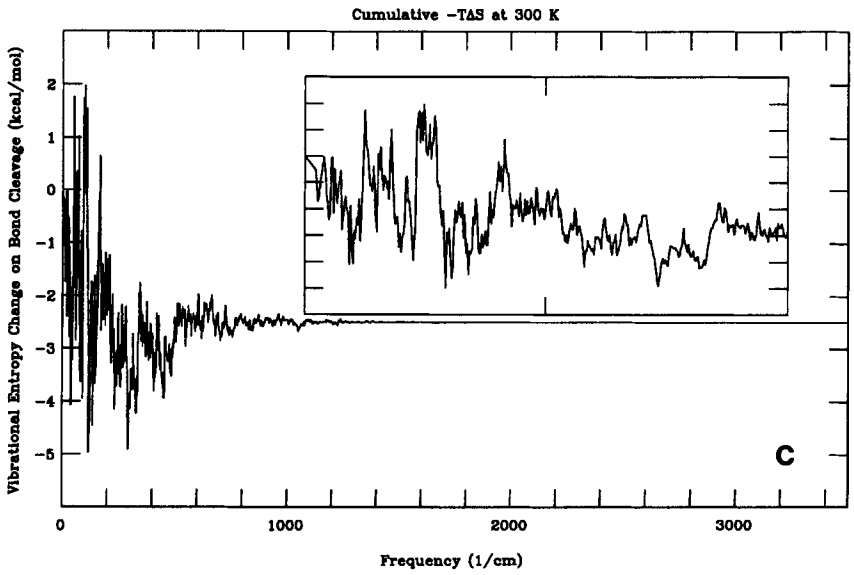

Cumulative -TΔS at 300 K
Vibrational Entropy Change on Bond Cleavage (kcal/mol)
2
1
0
-1
-2
-3
-4
-5
0
1000
2000
3000
Frequency (1/cm)
c

Fig. 1a–c.

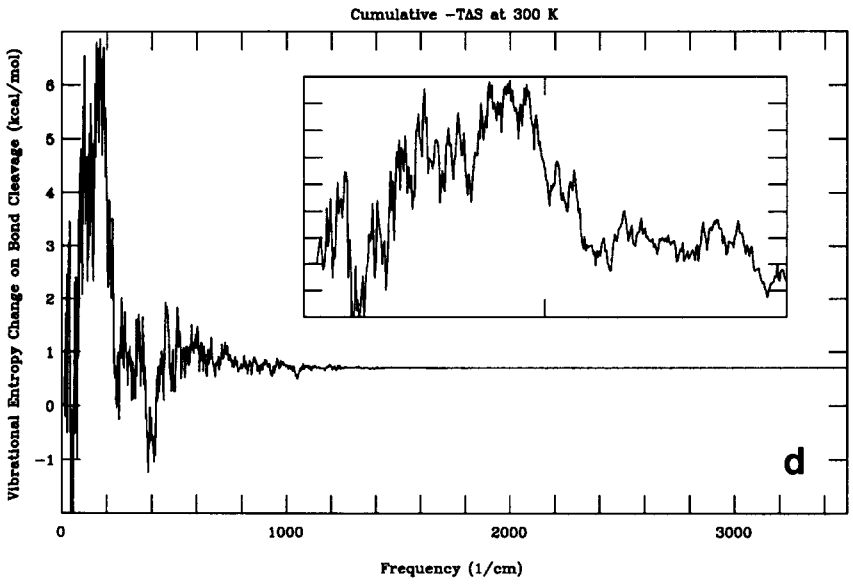


Fig. 1. The cumulative free energy change ($-T\Delta S$) due to the vibrational entropy difference between variant and native folded forms of BPTI. The inset shows an enlargement of the low-frequency region; the tick marks on the inset correspond with those on the main figure, starting from the left-hand edge. (**a**) the [30–51,5–55] variant; (**b**) the [14–38,5–55] variant; (**c**) the [14–38,30–51] variant; (**d**) the hydrolyzed variant.

disulfides causes a shift to smaller frequencies in the low-frequency spectrum and a corresponding increase in the vibrational entropy (i.e., the added entropy stabilizes the reduced species) due to the greater motional freedom of the folded state. This effect is in the range of 2.5–5.1 kcal/mol at 300 K. It should be noted that because the extended atom representation is used, an SH group is represented as a single atom; therefore, there is no change in the total number of degrees of freedom in the system upon reduction of disulfides in this model. Hydrolysis of the 15–16 peptide bond breaks a covalent bond, but replaces it with an ionic bond. Although there are nine additional normal modes in the hydrolyzed model (because hydrolysis adds one water molecule with three atoms or nine degrees of freedom), they are of high frequency and make a negligible contribution to the entropy. In contrast to the effect of reducing any of the three disulfides, the low-frequency spectrum for breaking the 15–16 peptide bond does not change significantly, and the entropic contribution to the free energy change is small and in the opposite (unfavorable) direction (+0.7 kcal/mol at 300 K). Figure 1a–d shows the convergence of the entropy difference for each bond-broken structure relative to native as a function of the maximum frequency included in the summation in Eq. (1). In each case the entropy difference is a result of contributions from modes up to 1000 cm^{-1}, but for the reduction of disulfide bonds, the largest contributions are due to changes in the lowest-frequency region of the spectrum (below 100 cm^{-1}). These are the global modes involving concerted motion throughout the molecule.[41] The inset shows that there is some correlation among the various species but that it is not complete. By contrast, the total entropy converges more slowly and has larger contributions from higher frequency modes (see Fig. 2).

Table III gives the relative change in the mean-squared fluctuations due to bond cleavage both for the atoms involved in cross-links and also for all atoms in the molecule. Significantly more freedom of motion results for atoms involved in disulfide bonds when these bonds are cleaved, but a significant restriction in the motion of the new C- and N-termini results from hydrolysis of the 15–16 peptide bond (due to the strong interaction and less space available because of added atoms). Although changes in local flexibility are significant for disulfide reduction (10–150%, averaging 45%), the overall flexibility (i.e., averaged over all the atoms) of all five molecules is virtually identical, except for [30–51, 5–55], which exhibits a 13% increase. Moreover, reduction of the 14–38 disulfide does not affect the flexibility of the carbonyl carbon of Lys-15, and cleavage of the 15–16 peptide bond does not change the amplitude of motion of the sulfur atoms of Cys-14. The implication is that predominantly local motional effects result from alterations in the global modes of the molecule, i.e., a sum over global modes is involved that leads to a local effect.

The normal mode calculations are useful because

TABLE II. Difference in Contribution of Vibrational Entropy to Free Energy From Native Structure at 300 K

Model	$-T\Delta S$ (kcal/mol)
1. Native	—
2. [30–51, 5–55]	−5.1
3. [14–38, 5–55]	−2.7
4. [14–38, 30–51]	−2.5
5. Hydrolyzed	+0.7

they allow the estimation and analysis of a single effect (i.e., the change in folded state vibrational entropy upon removal of a protein cross-link) on the stability of the protein. A complete analysis of all contributions would involve other entropic, as well as enthalpic, effects on both the folded and unfolded state, including solvation, conformational energy, and chain entropy in the unfolded state. The latter, in particular, is very difficult to quantify because of the long time scale and global nature of motions of the unfolded chain. Here the folded state vibrational contribution to the entropy has been calculated and is seen to contribute significantly to the overall stability change due to removing cross-links.

Of the three disulfides, 14–38 has the most effect on the entropy of the folded state, followed by 30–51 and 5–55. This is in accord with their positions in the molecule; i.e., 14–38 is at the top of an extended part of the molecule while both 30–51 and 5–55 are in the core where constraints are present. This ordering considers only the entropic contribution to the folded state. Even so, the motional data alone produce the ordering of disulfide stability observed for the folded molecule by measuring the effective concentration for each cysteine pair in the native state.[29] This suggests that the "entropic strain" may be more significant than the "enthalpic strain" of disulfides introduced into proteins, or perhaps, that the two are correlated. The equilibrium constant for hydrolysis of the 15–16 peptide bond has been measured experimentally,[37] and it is close to unity. This is interesting in light of our calculation of very little entropy difference between the states.

To evaluate the relative stability of each two-disulfide and the hydrolyzed form of BPTI (and to compare with available experimental results[32,38]) requires consideration of the effect of each cross-link on the unfolded state. Estimates based on random polymer statistics indicate that none of the cross-links removed in this study is responsible for more than a 3 kcal/mol free energy destabilization due to the effect of the cross-link on the entropy of the unfolded state.[4] Although this is a very simple model calculation, the likely error is no more than a factor of two. Consideration of excluded volume has led to a more detailed description of the effects of cross-links in the unfolded state,[6,7] but sequence-specific effects could also be significant and need to be included.

The hydrolysis result suggests the possibility of using a covalent cross-link to replace a noncovalent one to stabilize proteins. The hydrolysis of a peptide bond, resulting in charged ammonium and carboxyl groups, could lead to many changes in the folded state, including changes in solvation and electrostatic energy. To compare with the disulfide results, we have considered only the contribution of the vibrational entropy from the hydrolysis of the peptide bond. Cross-links introduced to stabilize proteins are usually thought of as primarily limiting the conformational freedom of the unfolded state so the change in going to the folded state is less unfavorable in terms of entropy. While this shifts the equilibrium toward the folded state, it is often offset by steric strain (added in greater measure to the folded than to the unfolded state) and reduced internal entropy in the folded state. If the cross-link can be added either in low-mobility regions of the molecule or between groups that are already noncovalently bonded (by ionic or hydrogen bonds), the destabilizing contribution from "motional strain" can be reduced. Even where this can be achieved, the new cross-link may not be stabilizing. The amino- and carboxy-termini of BPTI are close together[51] and form a salt bridge in solution.[55] Goldenberg and Creighton introduced a peptide bond between the termini to form a circularized BPTI molecule,[19] which was not more stable than the native protein. It would be useful to have temperature-dependent measurements to separate the enthalpic and entropic contributions in this case. But the decrease in stability is presumably due to some form of strain (entropic or enthalpic) introduced by the cross-link in the folded state.

Experimental attempts to engineer more stable proteins by the introduction of disulfide cross-links through site-specific mutagenesis have suggested the opposite conclusion. The greatest stability gains in T4 lysozyme were realized for disulfide bonds inserted into flexible regions of the structure.[27] The suggestion is that flexible portions (e.g., loops, chain termini, hinge regions) are better able to adjust and allow the disulfide bond to adopt an unstrained geometry.[27] That is, to avoid enthalpic strain in the folded state, a disulfide bond should be introduced in flexible regions of the molecule, but to avoid entropic strain it should be inserted in more rigid regions. Estimates of the two types of strain are of the same order of magnitude (compare Table II with enthalpic strains[27] of 4–8 kcal/mol). Further, they are of the same order as estimates of the unfolded state contribution,[16] and as the overall experimentally measured effect of disulfide-bond formation on protein stability (1–3 kcal/mol).[27] Thus, the contribu-

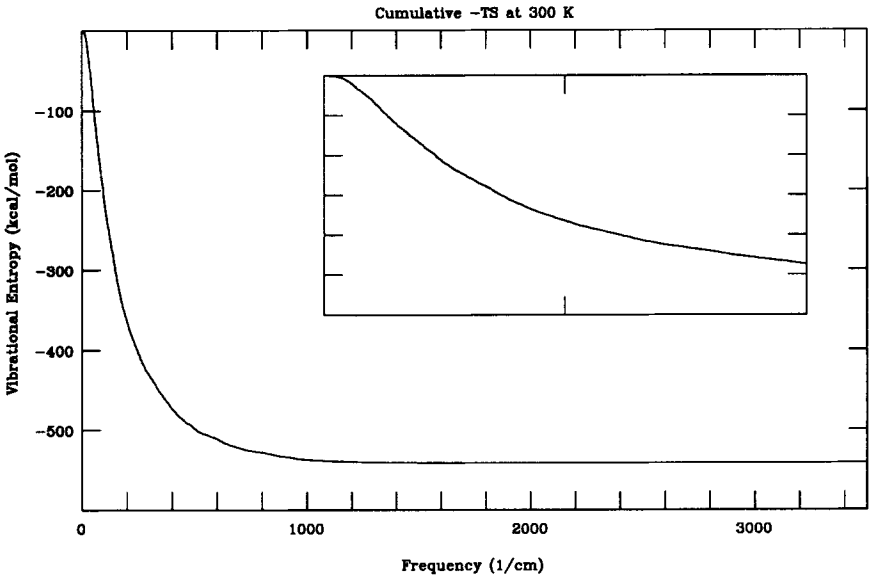


Fig. 2. The cumulative free energy ($-T\Delta S$) due to vibrational entropy in native BPTI. The inset shows an enlargement of the low-frequency region. The tick marks on the inset correspond to those on the main figure, starting from the left-hand edge.

TABLE III. Ratio of Mean-Squared Fluctuation Between Bond-Broken and Native Structures

Model	14 S_γ	38 S_γ	30 S_γ	51 S_γ	5 S_γ	55 S_γ	15 *C*	16 *N*	All atoms
1. Native	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2. [30–51, 5–55]	**1.10**	**1.71**	1.12	1.02	0.75	0.85	1.01	1.02	1.13
3. [14–38, 5–55]	0.99	0.98	**2.47**	**1.80**	0.92	0.96	1.03	1.04	1.03
4. [14–38, 30–51]	1.04	1.03	1.19	1.10	**1.31**	**1.22**	0.98	0.98	1.04
5. Hydrolyzed	1.05	1.01	1.13	1.28	0.80	1.13	**0.90**	**0.82**	0.97

tions of folded-state enthalpy and entropy as well as the unfolded-state chain entropy are all significant.

From the above results, it appears that it is a subtle balance between various factors that determines the overall effect of a disulfide bond on protein stability. Everything being equal, maximum entropic stabilization is expected for disulfide cross-links introduced far apart in the primary sequence (to maximize the effect in the unfolded state) but with near-optimum geometry and nature of the environment in the folded state. However, the effect on the internal motions as determined by the local rigidity must also be considered. Finally, plasticity, which refers to the ability of a region of protein structure to adapt to structural change,[56–58] can also play a role. For example, a protein hydrophobic core has liquid-like local motional properties[59] that allow it to adjust to small structural perturbations, but is constrained enough that a cross-link can be accommodated at modest entropic cost, as is the case for disulfides 30–51 and 5–55 in BPTI.

In an attempt to obtain information about the constraining effects of disulfides, Matthews and coworkers[12] have compared the refined crystal structure of a pseudo wild-type T4 lysozyme (Cys 54 → Thr and Cys 97 → Ala) with that of a disulfide-bridged mutant (the pseudo wild-type plus Ile-9 → Cys and Leu-164 → Cys). An examination of *B*-factors for the backbone atoms of these two structures reveals little difference between them, whereas our Table III shows restriction in the motion of sulfur atoms upon cross-link formation, though the atomic fluctuations averaged over the entire structure were indistinguishable. It would be useful to have an X-ray structural comparison of the oxidized and reduced forms of the mutant, and to look at the side chain *B*-factors—particularly those of the sulfur atoms involved in the cross-link.

CONCLUSION

The addition of intramolecular cross-links can substantially reduce the internal entropy of a protein molecule in the folded state. For the bovine pancreatic trypsin inhibitor, values of 2.5–5.1 kcal/mol were calculated for each of the three disulfides; this is of the order of the effect of the disulfides in the unfolded state. No such entropy loss resulted from peptide hydrolysis, suggesting that the introduction

of a cross-link to replace a strong noncovalent interaction might be used to achieve a destabilizing effect on the unfolded state without significantly affecting the entropy of the folded state. Engineered disulfides might be used in this manner, in low-mobility regions of proteins, to increase stability.

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