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Barnase thermal titration via molecular dynamics simulations: Detection of early denaturation sites

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Abstract

The thermal stability of barnase has been studied using constant pressure and temperature (CPT) molecular dynamics at different temperatures. Barnase X-ray coordinates were obtained from the *Research Collaboratory for Structural Bioinformatics* (RCSB) Protein Data Bank (PDB code:1rnb). Simulations were performed at 285, 295, 300, 335, 345, and 395 K in explicit water under periodic boundary conditions for 280 ps. For each simulation, conformations were saved every 0.2 ps. *Root mean square deviation* (RMSD) values were calculated relative to the starting structure at 300 K and at time $t = 0$. *Root mean square fluctuation* (RMSF) values were calculated relative to the average structure obtained from the 300 K simulation. Both root mean square deviation and fluctuation analysis indicated the presence of discrete regions of hyper-sensitivity along the barnase polypeptide chain. These regions exhibited spikes in flexibility prior to any global structural changes. The specific changes in barnase backbone flexibility are accompanied by increased phi/psi angle fluctuations. These results suggest the presence of early denaturation sites or denaturation nuclei whose local structure is disrupted prior to global structure disruption. Identification of denaturation nuclei suggests that appropriate amino acid replacements at these sites may lead to the design and development of more stable barnase mutants. This strategy of identifying denaturation nuclei in protein structures may represent a first step in the design of more stable protein structures.

Keywords: Barnase; Molecular dynamics; Thermal denaturation; Protein design

1. Introduction

Comparisons of homologous proteins from thermophiles and mesophiles have shown that related proteins can perform similar functions, yet have very different stabilities [1–4]. Therefore, the design of mutant proteins with increased stability and similar biological function relative to their wild type counterparts seems promising. Although native proteins undergo dynamic structural fluctuations [5–8], the overall global conformation of proteins is generally stable in the physiological temperature range. However, under extremes of thermal stress, proteins unfold and ultimately denature [9,10]. The rate and extent of denaturation generally occurs in response to the rate and severity of the thermal stress that is applied. The gradual unfolding of native proteins which results from gradual or incremental increases in thermal stress may lead to a more complete understanding of the mechanism(s) of protein denaturation and provide new insights into the design of proteins with enhanced thermal stability.

Barnase (E.C.3.1.2.7) from *Bacillus amyloliquifaciens* (MW, 12,402) which does not contain any prosthetic groups, metal cofactors, or disulfide bridges was chosen for study. Prosthetic groups, metal cofactors, and disulfide bridges all contribute to protein stability. However, the objective of this study was to investigate the contribution(s) of non-covalent intramolecular interactions to protein stability and how changes in these interactions are correlated with the initial events associated with tertiary structure unfolding in response to thermal stress. It was of interest to identify those segments along the barnase polypeptide chain which initially respond to thermal stress. These segments may represent areas of potential amino acid replacement with the goal of generating barnase mutants with increased thermal stability.

Studies reported here describe molecular dynamics simulations of barnase at 285, 295, 300, 335, 345, and

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395 K and the subsequent calculation of root mean square deviation (RMSD) and root mean square fluctuations (RMSF) values for the backbone atoms at each simulation temperature. These studies are designed to detect initial specific increases in backbone atom motion associated with increased thermal stress. Such thermally hyper-sensitive segments along the barnase backbone may represent candidates for further structural stabilization via amino acid replacement resulting in the design of barnase mutants with increased thermal stability.

2. Methodology and calculations

The X-ray coordinates of barnase were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB code:1rnb) [11]. The bound *guanylcytidyl di-ribonucleotide* (GC) substrate inhibitor was removed from the structure and hydrogen atoms were added using the HBUILD facility in CHARMM [12]. The resulting barnase structure was minimized in vacuo without constraints to a gradient RMS (GRMS) of 0.0028 kcal mol^{-1} $Å^{-1}$ using the Adopted-Basis Newton Raphson (ABNR) algorithm in CHARMM. During minimization and molecular dynamics simulation procedures, a non-bonded cutoff distance of 15 Å was used in conjunction with 13 and 14 Å, respectively, for switching on and off the van der Waals smoothing function. After minimization, the barnase structure was hydrated in a water box consisting of 5015 water molecules forming a 10 Å thick water shell around the protein. The three center polarizable, TIP3P water model of Jorgensen et al. [13] was used. The IMAGE facility of CHARMM was used to setup the periodic boundary conditions. The resulting solvated structure was further minimized using ABNR for another 300 steps followed by an additional 80 steps of ABNR minimization for adjustment of the crystal structure lattice. Molecular dynamics simulations were performed under constant pressure and temperature (CPT) conditions for 280 ps using the Leap-Frog integrator method in CHARMM. CPT production dynamics was preceded by 30–40 ps of non-CPT heating dynamics without SHAKE constraints to bring the system to the desired temperature. During CPT production dynamics, a typical SHAKE value of 1.0×10^{-5} was used. The Ewald summation method was used for calculation of Coulomb-interactions [14,15]. During the simulations, coordinates were saved every 0.2 ps. The simulations were performed using CHARMM, Version 27, running on four processor Silicon Graphics origin200 Servers. Simulations were performed at 285, 295, 300, 335, 345, and 395 K. For each simulation, the barnase structure at time $t = 250$ ps was analyzed in comparison with the structure at time $t = 0$ ps and at 300 K (starting structure).

Hydrogen bonds present in the hyper-flexible regions were calculated and visualized using the Hbond facility in the Quanta molecular modeling software package from Accelrys Inc. Quanta was also used to visualize the conformations of these regions and the surface-exposed residues of the barnase structure.

The root mean square deviation for the simulation structures at time $t = 250$ ps relative to the starting structure was calculated according to the following expression:

$$\mathrm{RMSD} = \sqrt{\frac{\sum_i (x_i - X)^2}{n - 1}}$$

where x_i refers to the position of residue i at time $t = 250$ ps. X refers to the position of the corresponding residue i in the starting structure at time $t = 0$ and 300 K and n refers to the total number of residues in the barnase molecule. For root mean square fluctuation calculations, X is replaced by the average position of residue i during the 300 K simulation. The value of X is calculated according to the expression below:

$$X = \frac{1}{n}\sum_j x_i$$

In this case, x_i is the position of residue i at time $t = j$ during the simulation and n corresponds to the total number of time points. Residue RMSD and RMSF calculations were performed only on the polypeptide chain backbone atoms.

The ConSurf-HSSP database [16] was used to obtain conservation grades for each amino acid position along the barnase sequence. The ConSurf-HSSP database has been generated from the related Homology-derived Secondary Structure of Proteins (HSSP) database [17]. The HSSP database contains multiple sequence alignments [18] for three-dimensional protein structures present in the Protein Data Bank [11]. The Rate4Site algorithm [19] uses multiple sequence alignment data from the HSSP database to generate conservation scores for each amino acid position in three-dimensional protein structures. The ConSurf-HSSP database contains conservation scores calculated by the Rate4Site algorithm for each amino acid position within each three-dimensional protein structure present in the HSSP database. The Rate4Site conservation scores may also be related to corresponding conservation grades. The ConSurf-HSSP database may be accessed at http://consurf-hssp.tau.ac.il or via the PDBsum website http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/. Finally, the ConSurf-HSSP amino acid conservation analysis for barnase can be accessed directly at the following web address: http://bioinfo.tau.ac.il/~consurf/results/HSSP_ML_1rnbA/index.htm.

3. Results

Following molecular dynamics simulations at multiple temperatures, it was of interest to determine which general regions of the barnase polypeptide chain exhibit hyper-sensitivity to thermal stress. Consequently, instead of analyzing RMSD behavior of individual residues, initial backbone RMSD analyses were performed on groups of 5 residues along the polypeptide chain. The RMSD behavior of these residue groups in response to increases in thermal stress should give an indication of those regions of the barnase polypeptide chain which are most sensitive or responsive to heating. These results are shown in Fig. 1. As can be seen, there is a significant

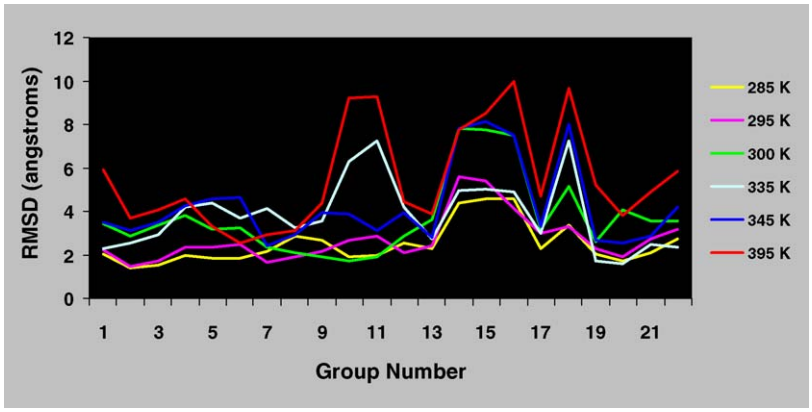


Fig. 1. Comparison of RMSD behavior of 5-residue groups along the barnase polypeptide chain at 285, 295, 300, 335, 345, and 395 K.

temperature-dependent increase in RMSD values between segments 9 and 12 which approximately corresponds to residues 45–60. There were also temperature-dependent RMSD increases between segments 13 and 17 and between 17 and 19 which correspond to residues 65–85 and 85–95, respectively. More moderate increases are observed at both the amino- and carboxy-terminii. These observations indicate that temperature-dependent increases in flexibility occur primarily in the central and carboxy regions on the barnase molecule.

It was of further interest to investigate the temperature-dependent RMSD changes along the barnase backbone as a function of individual residue position. These results are shown in Fig. 2. It is immediately recognized (300 K data) that the region between residues 55 and 73 possesses significantly more native flexibility than is observed for other portions of the barnase chain. As the temperature increases, these peaks generally become more pronounced with the appearance of new peaks at residues 35–40 and 73–85. This pattern is especially noted in the 345 and 395 K simulations. The data from the 395 K are dominated by a large new peak at position 38. The 395 K data also exhibit a smaller but certainly prominent new peak at position 7. The data in Fig. 2 clearly identify several regions of temperature-induced hyper-flexibility along the barnase chain. These regions are positions 7, 35–43, 64–70, and 73–88. Less prominent increases occur in the region between residues 90 and 99. When viewed collectively, the data from Fig. 2 indicate that in the amino-terminal region, there occurs a large increase in RMSD values over a restricted range of amino acids (35–43), while in the carboxy-terminal region, several portions of the chain exhibit temperature-dependent changes in RMSD values. In the amino-terminal region, the changes occur in a region of baseline-like RMSD values, while in the carboxy-terminal the changes occur over a background that is naturally more flexible. These observations clearly reveal the different behavior of the amino- and carboxyl-terminii portions of the barnase molecule in response to increasing thermal stress.

Since RMSD calculations may also include atomic translational effects due *to* changes in molecular orientation as a result of rotational or translational motion, a change in orientation could mask the effect of actual conformational change or unfolding. In order to eliminate this possibility, it was decided to calculate the RMSF of the backbone atoms. In order to accomplish this, the average structure for barnase during the 300 K simulation was calculated. This average structure was

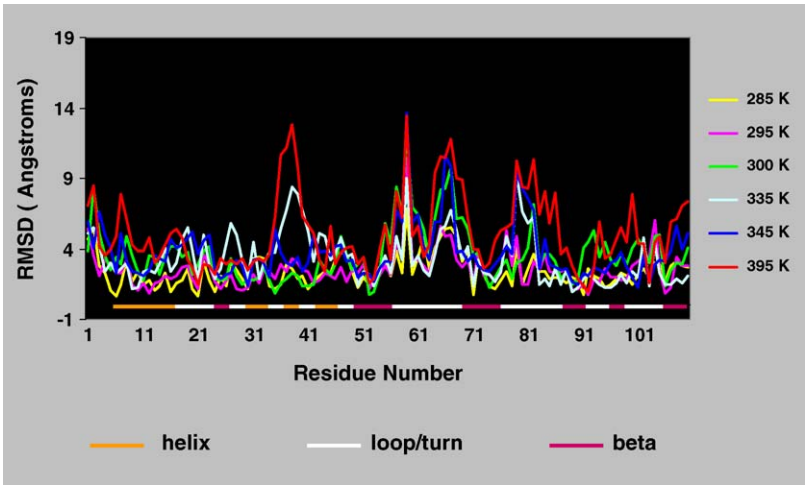


Fig. 2. RMSD behavior of backbone atoms along the barnase polypeptide chain at 285, 295, 300, 335, 345, and 395 K. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

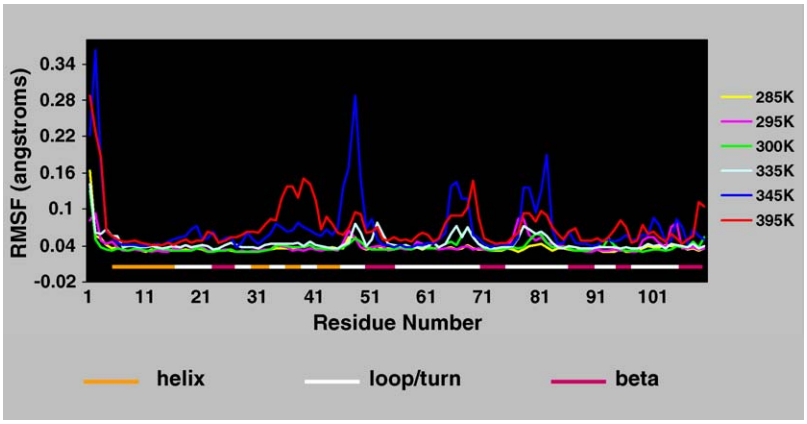


Fig. 3. RMSF behavior of backbone atoms along the barnase polypeptide chain at 285, 295, 300, 335, 345, and 395 K. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

then utilized in conjunction with the $t = 250$ ps structure from each simulation to calculate the RMSF values of backbone atoms. However, prior to calculating RMSF values, each $t = 250$ ps structure and the average structure at 300 K was placed in identical molecular orientations using the coordinate orientation facility in CHARMM. The resulting RMSF data are shown in Fig. 3. A relatively flat baseline is seen for the structures at 285, 295, and 300 K. However, there appears to be a small bump-like peak in the vicinity of position 48. At 345 K, the plot is dominated by large peaks at positions 48 and 66, and split peaks at 79 and 82. Additionally, the peak at 66 is broad with a pronounced shoulder at 69. At 395 K, a new double peak appears at positions 36 and 40, while the large peak at position 48 in the 345 K simulation is greatly diminished. Also, at 395 K, the shoulder peak at 69 in the 345 K simulation has become the major peak with a shoulder at position 66. The twin peaks observed at positions 79 and 82 in the 345 simulation continue to be present but they are much smaller. It is very interesting that there is apparent linear shifting of flexibility along the barnase polypeptide chain between the 345 and 395 K simulations. For example, when these two simulations are compared, it appears that a region of hyper-flexibility centered at position 48 in the 345 K structure is shifted to positions 36–40 in the 395 K structure. Likewise, a peak and shoulder at 66 and 69, respectively, at 345 K have become a shoulder at 66 and peak at 69 in the 395 K simulation. These observations suggest internal structural rearrangements in the barnase molecule in response to thermal stress. Consequently, as these structural rearrangements occur, some regions of the barnase structure may experience transient increases or decreases in RMSF values. The data in Fig. 3 clearly demonstrate the presence of discrete regions or 'hot spots' along the barnase backbone that respond in a hyper-sensitive manner to increased thermal stress. These hyper-flexible regions occur at residues 35–42, 46–50, 64–71, and 78–83. Additionally, it can be seen from Fig. 3 that the hyper-flexible regions all occur in loop structures, except for region 35–42, which consists of a loop–helix–loop motif. The response of these regions to increased thermal stress is suggestive of a higher level of intrinsic flexibility along these segments of the barnase polypeptide chain.

In order to gain insight into the natural flexibility of the barnase sequence, the C–α temperature factors obtained from the barnase X-ray crystal structure (PDB code:1rnb) were plotted as a function of residue number. These results are shown in Fig. 4. As can be seen, while the location of the hyper-flexible regions or 'hot spots' is associated with peaks or shoulders, it is clear that the 'hot spot' regions of the chain do not exhibit temperature factor values that appear unusually greater than other segments of the barnase chain. In fact, some segments of the barnase sequence exhibited temperature factor

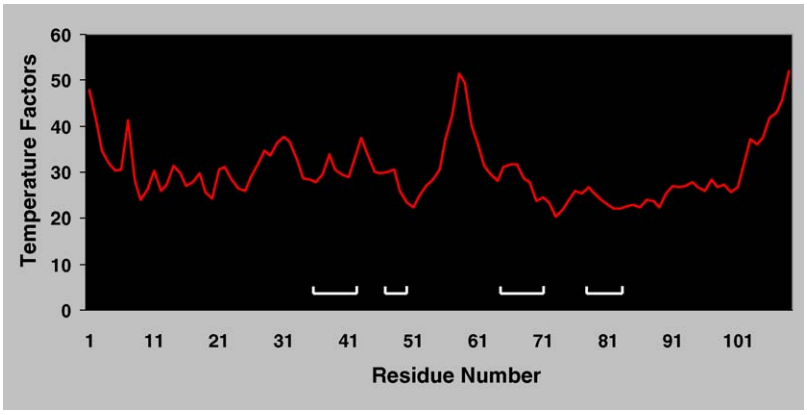


Fig. 4. Analysis of barnase C–α temperature factors as a function of residue position. White brackets indicate positions of hyper-flexible regions.

values quite larger than those exhibited by the 'hot spot' regions. Consequently, these data suggest that the observed 'hot spots' may be better described as a position or sequence-dependent response to thermal stress than an expression of localized intrinsic flexibility in the non-stressed molecule.

Hydrogen bonding is an important contributor to protein stability. The hydrogen bonding pattern for each of the 'hot spot' regions from the simulations at 300, 345, and 395 K and time $t = 250$ ps are shown in Fig. 5a–d, respectively. First, it should be noted that in most cases, these segments of the barnase chain undergo changes in conformation and in hydrogen bonding pattern as a function of temperature. While changes in conformation and hydrogen bonding naturally occur in proteins, it is likely that the changes observed in Fig. 5 were related to the application of thermal stress to the barnase structure. Conformational changes are especially noted in the

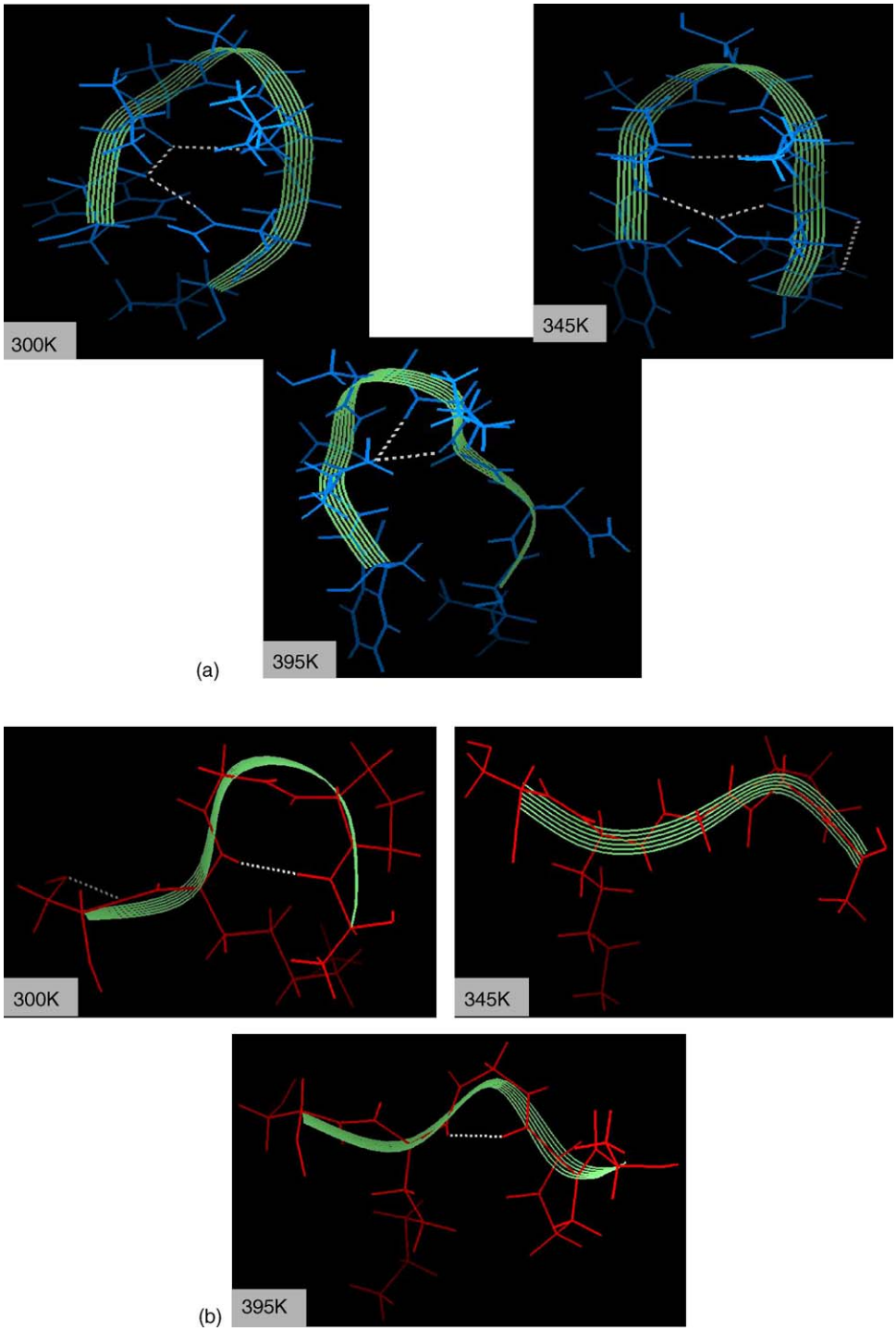
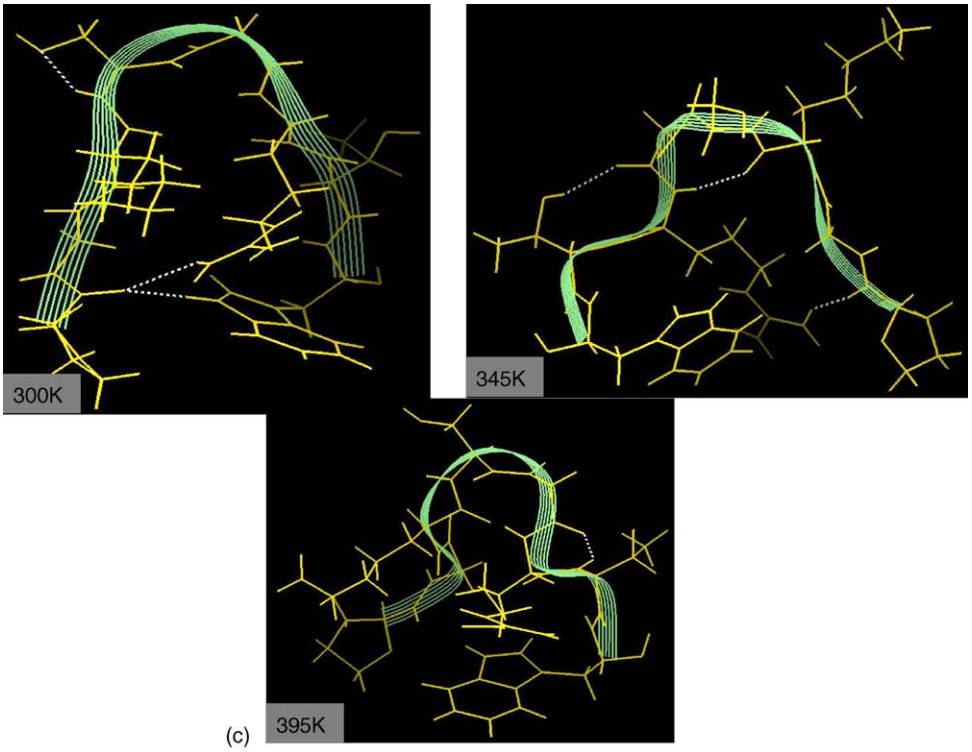


Fig. 5. Conformation and hydrogen bonding pattern of barnase thermally induced hyper-flexible (hot spot) regions: (a) 35–42, (b) 46–50, (c) 64–71, and (d) 78–83. Green ribbons indicate backbone conformations of hyper-flexible regions.



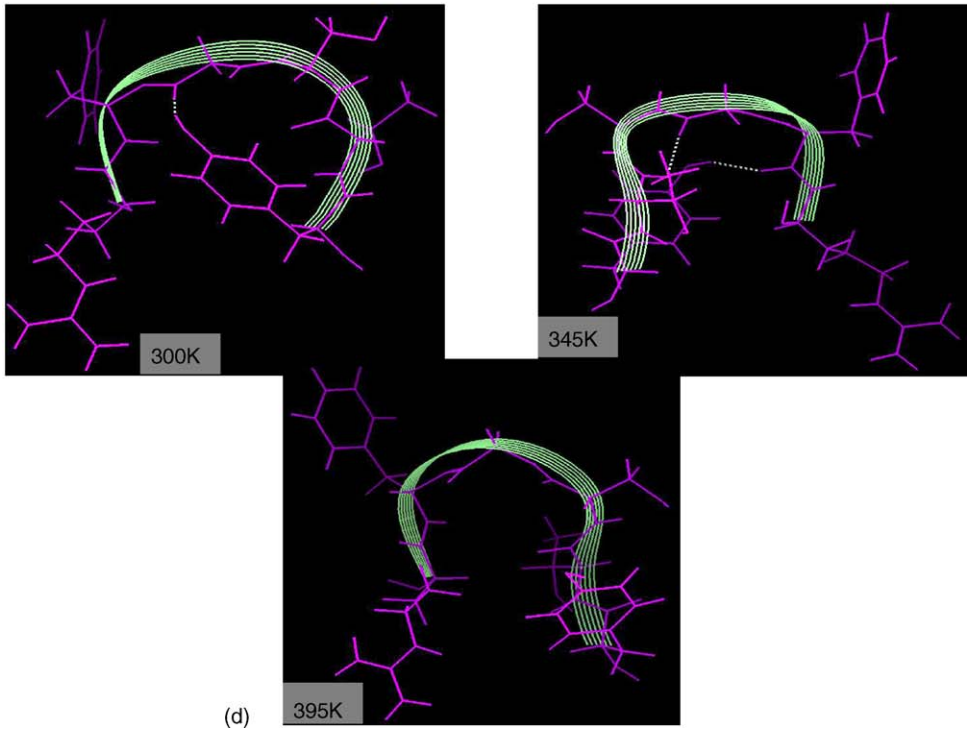


Fig. 5. (*Continued*).

hyper-flexible region corresponding to residues 46–50 (Fig. 5b). At 300 K, this region appears as a well-defined loop with one hydrogen bond. At 345 K, it is highly extended with very little defined structure with no hydrogen bonds, and at 395 K, it still appears relatively linear with a single hydrogen bond. It should be noted that this region displays its greatest flexibility at 345 K which corresponds to a local conformation without any hydrogen bonds. The combined 'hot spot' structures at 300, 345, and 395 K collectively exhibited nine, nine, and four hydrogen bonds, respectively. These observations suggest the gradual destabilization of the protein in concert with decreases in hydrogen bonding and increasing thermal stress.

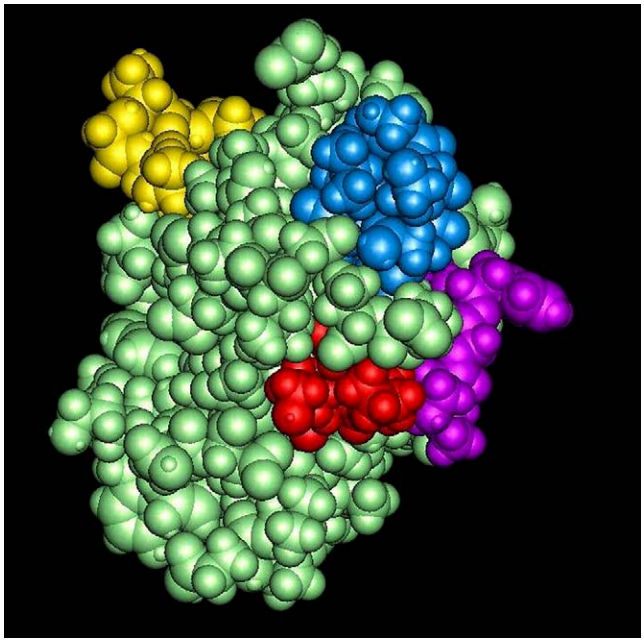


Fig. 6. Surface representation of the barnase structure illustrating the surface-exposure of hyper-flexible regions: region 35–42 (blue), region 46–50 (red), region 63–71 (yellow), and region 78–83 (purple).

Fig. 6 is a representation of the barnase structure which illustrates the surface- or solvent-exposed residues. As can be seen, each of the hot spot regions is highly solvent accessible. Since tryptic cleavage sites are often associated with solvent accessibility, the presence of tryptic cleavage sites in the 'hot spot' regions was investigated. Fig. 7 shows the barnase amino acid sequence. The 'hot spot' regions of the sequence are highlighted and tryptic cleavage sites within or adjacent to these regions are indicated by arrows. Each 'hot spot' is associated with at least one cleavage site and the region 64–71 contains two cleavage sites.

It was of interest to investigate the evolutionary conservation of the hyper-flexible or 'hot spot' residues. The ConSurf-HSSP database [16] was used to obtain the evolutionary conservation

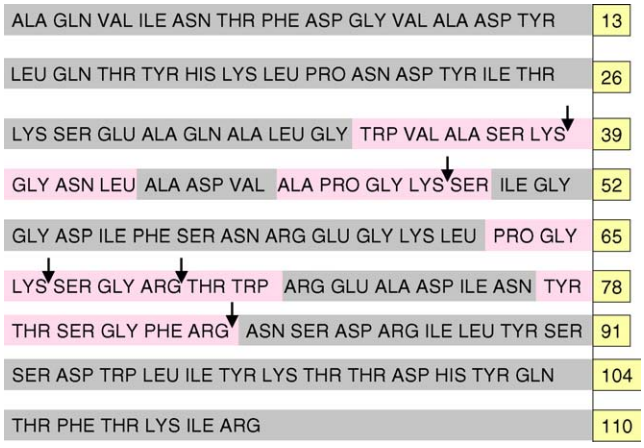


Fig. 7. The barnase amino acid sequence. Hyper-flexible (hot spots) are highlighted in pink. Arrows indicate positions of tryptic cleavage sites.

pattern for the barnase sequence. The resulting pattern is shown in Fig. 8. Conservation grades of 7, 8, and 9 are considered to reflect conserved residues, while grades of 1, 2, and 3 are indicative of residues that are not conserved. Generally, the residues in the amino-terminal half (residues 1–50) of the barnase molecule appear less conserved than those present in the carboxy-terminal portion (residues 51–110). It is interesting that two 'hot spot' regions occur in the amino-terminal portion (35–42 and 46–50) while two occur in the carboxy-terminal portion (residues 64–71 and 78–83). The region 46–50 showed the highest level of conservation with 100% of the amino acids in this segment exhibiting conservation grades of 7, 8, or 9. In the 35–42, 64–71, and 78–83 segments, only 25, 38, and 67% of the residues are conserved, respectively.

Fig. 9 shows the barnase phi and psi angle values during the 300 K molecular dynamics simulation. It can be seen that helical and beta portions of the chain exhibit phi/psi values that are predicted by Ramachandran plots for globular proteins [20,21]. These observations underscore the validity of the structural predictions during the simulation. Changes in protein conformation such as unfolding usually occur in concert with fluctuations in phi and psi angles. The phi and psi angle averages were calculated at 300, 345, and 395 K (Figs. 10 and 11, respectively). The general patterns for the phi and psi averages appear similar for each temperature. However, small differences were observed in the phi averages at positions 2, 43–47, 85–95, and 100–105. For the psi averages, small differences were noted at positions 42–45, 47–48, 65–70, 75, 80, 92, and 99–100. Consequently, it was of interest to investigate the temperature-dependent phi/psi angle fluctuations about the average values as a result of increased thermal stress. Figs. 12 and 13 show the phi and psi angle fluctuations, respectively. The phi angle fluctuations at 300 K exhibit peaks at several discrete locations that are distributed throughout the polypeptide chain. However, the psi angle fluctuation pattern at 300 K looks much different. The amplitude of the peaks is much larger and the vast majority of the peaks occur in the carboxy-terminal portion of the molecule. This observation suggests that the natural flexibility observed for the carboxy-terminal portion of barnase may be largely due to significant naturally occurring psi angle fluctuation in that portion of the molecule. In fact, the number of psi angle fluctuation peaks in the carboxy-terminal is more than twice that observed for the amino-terminal. The most striking feature of the 345 K simulation is the emergence of new phi and psi fluctuation peaks in the amino-terminal portion. The most notable feature of the carboxy-terminal is the increased amplitude observed for existing fluctuation peaks. In the 395 K simulation, there are significant decreases in phi peaks in the vicinity of positions 80 and 87 relative to the 345 K simulation. There is also a very large increase in the psi peak at position 33 when compared to the 345 K simulation. Collectively, Figs. 10–13 indicate that phi angle fluctuations generally increase from 300 to 345 K and decrease from 345 to 395 K, while psi angle fluctuations continue to increase as the temperature is increased. These observations likely suggest the presence of internal structural rearrangements in response to thermal stress. Additionally, it is

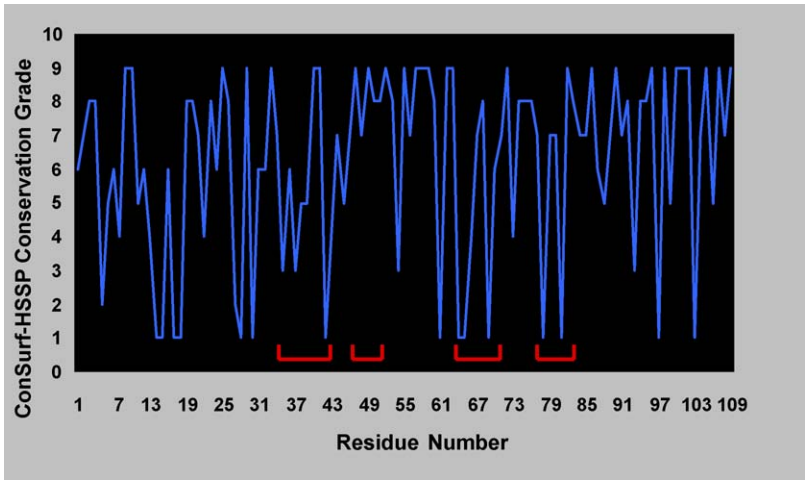


Fig. 8. The ConSurf-HSSP conservation pattern for the barnase amino acid sequence. Red brackets indicate positions of hot spot regions.

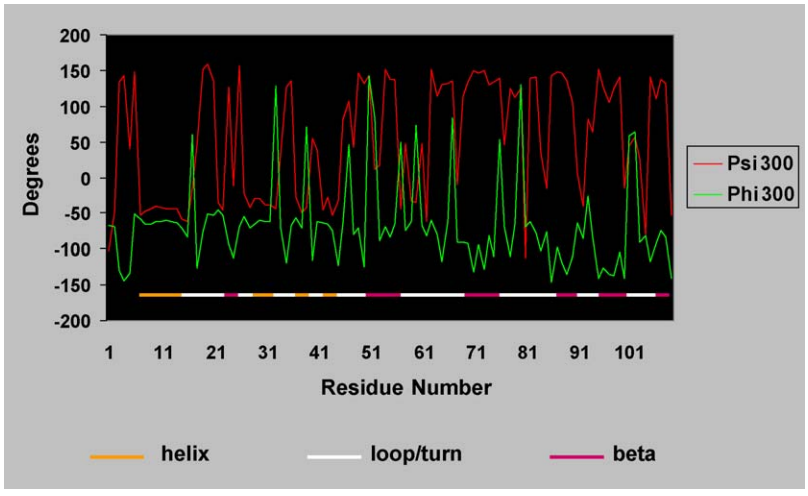


Fig. 9. Comparison of barnase phi and psi angle values during 300 K molecular dynamics simulation. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

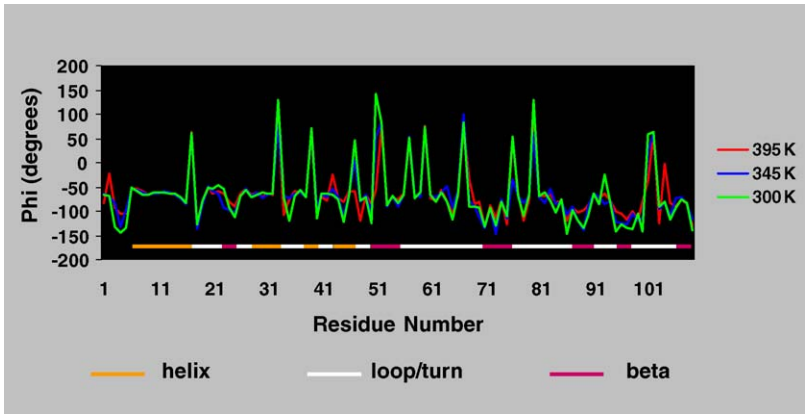


Fig. 10. Backbone phi angle averages at 300, 345, and 395 K for each barnase residue. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

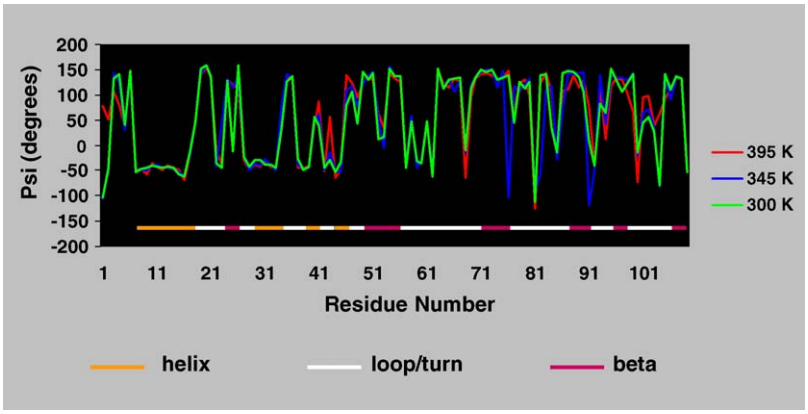


Fig. 11. Backbone psi angle averages at 300, 345, and 395 K for each barnase residue. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

suggested that in the case of barnase, the initial application of thermal stress stimulates increased fluctuations in both phi and psi angles, but as the stress is continually applied, psi angle fluctuations appear to dominate the structural adjustment. It should also be noted that the positions of the new and/or enhanced psi angle fluctuation peaks are distributed throughout the polypeptide chain and generally correspond to those regions which showed increases in backbone atom RMSF fluctuations. Observed differences in phi and psi angle fluctuations are reminiscent of recently reported differences in phi and psi angle

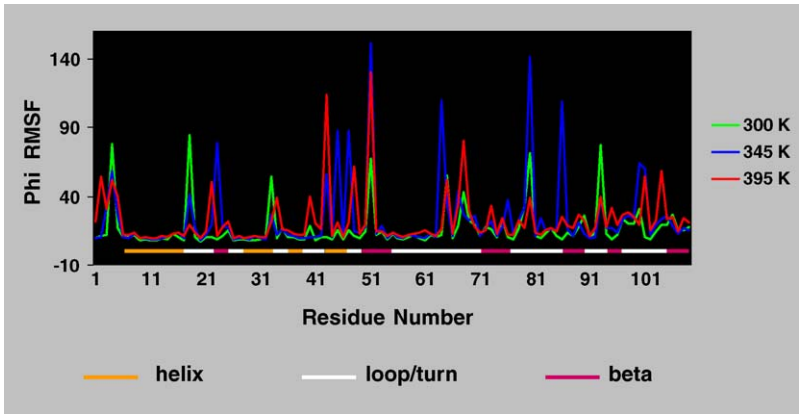


Fig. 12. Backbone phi angle fluctuations at 300, 345, and 395 K for each barnase residue. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

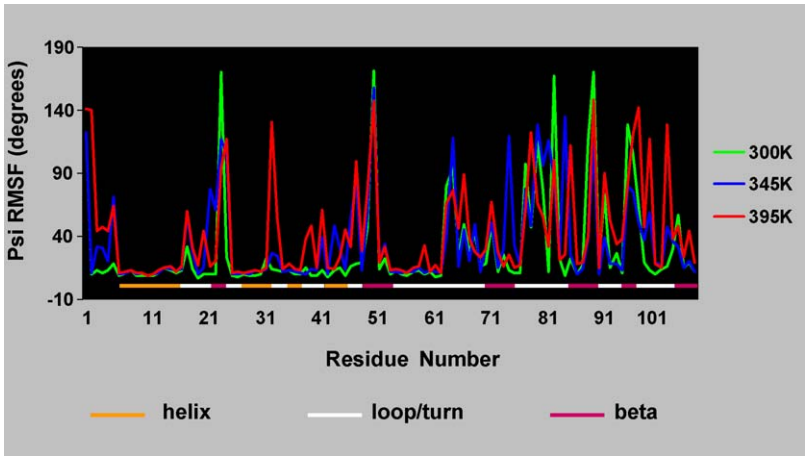


Fig. 13. Backbone psi angle fluctuations at 300, 345, and 395 K for each barnase residue. Helix and beta regions are indicated by orange and purple line segments. Other regions are generally referred to as loop/turn and are indicated by the white line segments.

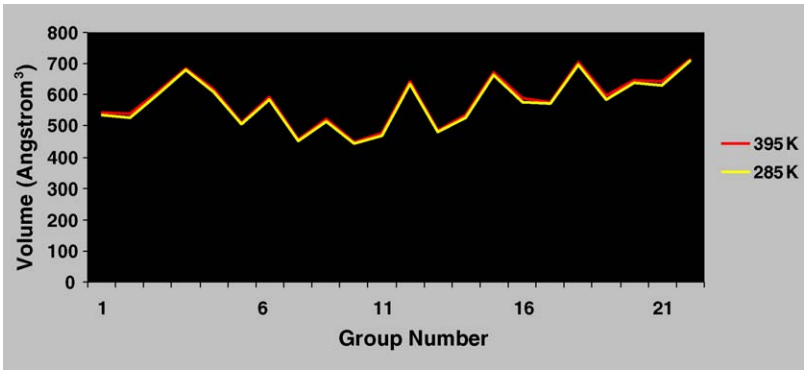


Fig. 14. Volume comparisons at 285 and 395 K for each barnase 5-residue segment.

transition rates [22]. It should be noted that differences in phi and psi angle transition rates are consistent with differences in phi and psi angle fluctuations. Fig. 14 shows that there were very minimal volume changes for each 5-residue segment of the barnase structure, which is consistent with the maintenance of overall structural integrity during the temperatures studied. Fig. 15 shows the backbone structures of barnase after the simulations at 300, 345, and 395 K. As can be seen, the native structure of barnase is essentially retained throughout these simulations. Consequently, changes observed in these studies reflect very early structural adjustments in response to thermal stress much before denaturation occurs.

4. Discussion

Fig. 1 immediately distinguishes the behavior of the amino- and carboxy-terminii regions of barnase in response to thermal stress. At 300 K, the amino-terminal region is essentially flat, while the carboxy-terminal region shows significant peaks. With increasing temperatures the amino-terminal regions shows a large peak at *group* 8. These results demonstrated the presence of natural flexibility in the carboxy-terminal region and thermally induced flexibility in the amino-terminal region.

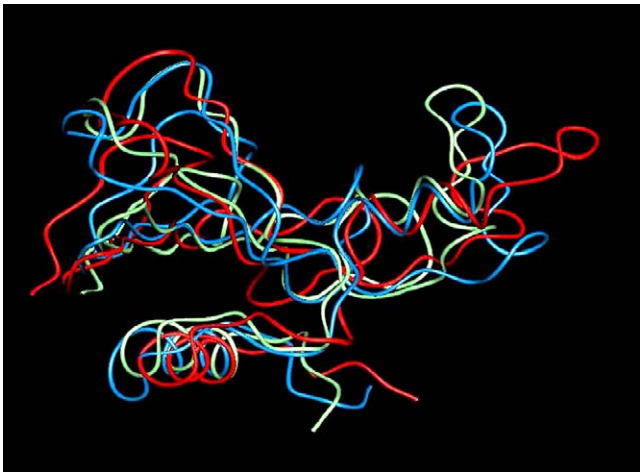


Fig. 15. Comparison of barnase backbone structural conformations at 300 K (green), 345 K (blue), and 395 K (red).

Figs. 2 and 3 show a more detailed effect of thermal stress on the backbone atoms of the individual residues. Both RMSD and RMSF calculations indicate significant temperature-dependent increases in the amino-terminal fluctuation between residues 30 and 45. In the case of RMSF calculations, the curve at 300 K appears generally flat except for residue 1 and for a small peak at approximately residue 47. On increasing thermal stress, very large peaks developed at specific sites in both the amino- and carboxy-terminii regions. The data shown in Figs. 2 and 3 suggest a position- or sequence-dependent response to thermal stress along the barnase polypeptide chain. Additionally, the shifting of peaks observed between the 345 and 395 K simulations suggests that there is an adjustment of local structure, such that a region which exhibits flexibility at 345 may be in a conformation at 395 K that limits it flexibility, while other regions with limited flexibility at 345 K may exhibit increased flexibility at 395 K.

In globular proteins loop regions are generally associated with surface- or solvent-exposed residues. Solvent-exposed regions are often associated with limited proteolysis sites. The barnase 'hot spot' regions primarily occur in loop regions that are surface-exposed and are associated with tryptic cleavage sites. However, it should be noted that region 35–42 is best described as a loop–helix–loop motif. Additionally, X-ray crystallography temperature factors (Fig. 4) indicate that these 'hot spots' generally do not exhibit any increased natural (unstressed) flexibility relative to other segments of the barnase polypeptide chain. Therefore, it appears that the observed hyper-flexible regions are due to position- or sequence-dependent responses to thermal stress. In general, the 'hot spot' regions exhibit varying levels of amino acid conservation, from 100% for the 46–50 region to 25% for the 35–42 region. Therefore, the hyper-flexible response to thermal stress is not likely mediated by the presence of conserved residues. The 'hot spot' regions appear to undergo thermally induced changes in conformation and hydrogen bonding pattern as observed in Fig. 5a–d. It is suggested that thermally induced destabilization of these regions may represent the initial steps in global destabilization of the barnase molecule.

Polypeptide backbone flexibility is closely aligned to the flexibility of phi and psi angles. Phi angle fluctuations showed well-defined peaks throughout the structure during the 300 K

simulation, while the corresponding psi angle fluctuations at 300 K were most prominent in the carboxy-terminal region. Both the phi and psi angle fluctuation patterns showed changes in response to thermal stress (Figs. 12 and 13). The phi angle response was primarily the appearance of new peaks, while the psi angle response consisted of the appearance of new peaks mostly in the amino-terminal region and an increase in existing peaks in the carboxy-terminal region. In both cases, the phi and psi angle responses seem to occur at sites which generally correspond to sites which exhibit RMSD and RMSF hyper-sensitivity. Collectively, results presented here indicate that specific regions along the barnase polypeptide chain exhibit hyper-sensitivity to thermal stress and that these regions respond to thermal stress at the very early stages of denaturation prior to the global disruption of the barnase structure. These sites may be considered denaturation nuclei and therefore may represent targets for amino acid replacement in the design of more stable barnase mutants.

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