Differential Stability of β-Sheets and α-Helices in β-Lactamase: A High Temperature Molecular Dynamics Study of Unfolding Intermediates

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ABSTRACT β-Lactamase, which catalyzes β-lactam antibiotics, is prototypical of large α/β proteins with a scaffolding formed by strong noncovalent interactions. Experimentally, the enzyme is well characterized, and intermediates that are slightly less compact and having nearly the same content of secondary structure have been identified in the folding pathway. In the present study, high temperature molecular dynamics simulations have been carried out on the native enzyme in solution. Analysis of these results in terms of root mean square fluctuations in cartesian and [ϕ, ψ] space, backbone dihedral angles and secondary structural hydrogen bonds forms the basis for an investigation of the topology of partially unfolded states of β-lactamase. A differential stability has been observed for α-helices and β-sheets upon thermal denaturation to putative unfolding intermediates. These observations contribute to an understanding of the folding/unfolding processes of β-lactamases in particular, and other α/β proteins in general.

INTRODUCTION AND BACKGROUND

Most globular proteins appear to attain their native structure through the formation of transient intermediates (Matouschek et al., 1990; Udgaonkar and Baldwin, 1990; Roder et al., 1988; Kim and Baldwin, 1990; Dobson, 1991; Englander and Mayne, 1992). Detailed experimental characterization of the molecular structure of these intermediates is problematic, since it is extremely difficult to isolate transient structures. Physical properties such as compactness, secondary structural content (Goto et al., 1990a, b), and the nature of side chain packing (Goto and Fink, 1989) have been determined in certain cases, and have proved useful in the characterization. However, there is not sufficient information from this approach for structure prediction.

Unfolding intermediates have recently become accessible to theoretical investigation by molecular dynamics (MD) simulation, and studies have now been reported on lysozyme (Mark and van Gunsteren, 1992), α-lactalbumin (Fan et al., 1991), BPTI (Daggett and Levitt, 1992a), and apomyoglobin (Tirado-Rives and Jorgensen, 1993). Transient conformations may be identified in high temperature MD runs, and their structural characteristics analyzed in detail in the context of available experimental data. MD simulations of the native structure at room temperature usually do not generate structural intermediates in the folding/unfolding pathway, since the length of the simulations are relatively short and the barriers required for the conformational transition to the unfolded state may not be thermally accessible. These problems can be transcended by heating the system to a reasonably high temperature, thus enabling the system to undergo transition to unfolding intermediates in a short period.

β-Lactamase from *Staphylococcus aureus* (PC1) is a single polypeptide chain of 257 residues, with a molecular mass 29,000 daltons. Compact intermediates in the folding pathway of β-lactamases have been experimentally identified (Goto et al., 1990a, b; Creighton and Pain, 1980; Thomas et al., 1983; Craig et al., 1985; Goto et al., 1990; Christensen and Pain, 1991), and possible structures for these forms are thus of interest. Solution studies using circular dichroism (CD) (Craig et al., 1985), urea-gradient gel electrophoresis, and sedimentation analysis have shown that these intermediates retain most of the secondary structure, and are less compact than the native form. The enzyme is made up of 14 helices and nine β-strands and is classed as an α/β protein (Branden and Tooze, 1991) (Fig. 1). It has no cysteines and thus the tertiary structure is governed only by noncovalent interactions. This characteristic makes β-lactamase especially suited for theoretical unfolding studies. β-Lactamase can be reversibly unfolded, and stable intermediates (states H and I) have been identified (Goto and Fink, 1989; Thomas et al., 1983).

In this paper, we report an extensive MD simulation study, including explicit water, of the unfolding of a class A β-lactamase from *S. aureus*. We generate putative intermediate states of β-lactamase and determine the intrinsic stability of secondary structural motifs as the protein unfolds. Their stability is measured by the extent of secondary structure retained within each region, and by the root mean square (RMS) fluctuation with respect to the crystal form in both cartesian and [ϕ, ψ] space. Analysis of the topology of partially unfolded states of β-lactamase in terms of secondary structures reveal a differential stability for helices and sheets upon thermal denaturation, with β-sheets motifs more thermally stable than α-helices. The differential stability for β-sheets and α-helices in β-lactamase provides some new perspectives on the protein folding problem.

METHODS

MD studies at high temperature (600 K) were carried out on β-lactamase native enzyme in solution. Room temperature (300 K) simulations were carried out to serve as a control.

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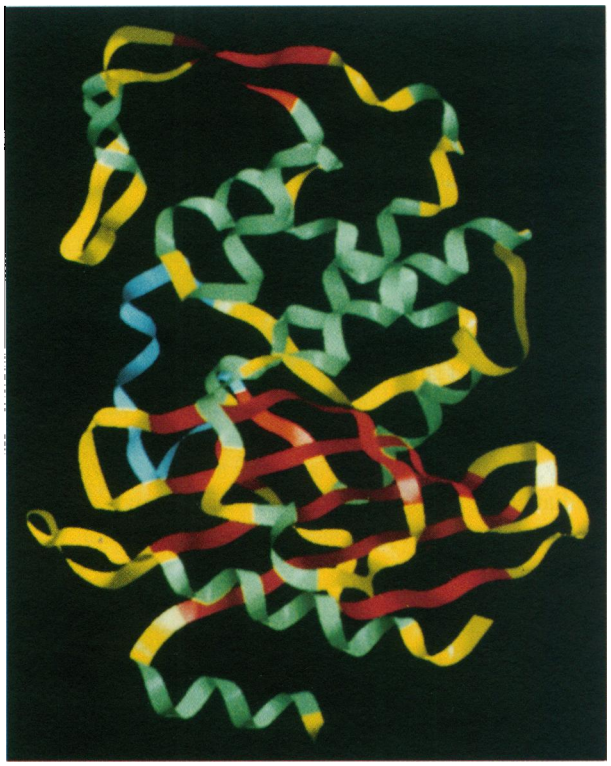

FIGURE 1 Ribbon drawing of β-lactamase from *S. aureus*. Secondary structures are color coded as follows; α-helices are *green*, β-sheets are *red*, omega loop is *blue*, and turns and loops are *yellow*.

Simulation of the native enzyme was started from the published crystal structure (Herzberg, 1991). Sixteen water molecules identified as internal from crystallographic analysis were included in both simulations. The simulations at both temperatures were carried out at constant volume and temperature. The protein was solvated with 7295 molecules of water in a hexagonal prism cell of height 71.28 Å with an inscribed circular radius of 34.57 Å, which corresponds to a bulk density of 1.0 g/cc. The use of these conditions is somewhat arbitrary, although similar to conditions previously employed (Mark and van Gunsteren, 1992; Daggett and Levitt, 1992a) and has been carried out as a preliminary study to make unfolding accessible within the timescale of MD simulations. However, comparison of the putative intermediates from MD with those identified experimentally suggests that the effect of simulation conditions employed herein may not be very severe within the picosecond time scale.

Molecular dynamics calculations in this study were performed with the MD simulation program WESDYN (Swaminathan, 1990; Ravishanker et al., 1993) using the RT37C4 force field of GROMOS86 (van Gunsteren and Berendsen, 1986). The GROMOS forcefield employs an explicit hydrogen atom model for polar groups and a united atom model for nonpolar groups. Switching functions were used to make the long-range nonbonded interactions go smoothly to zero between 7.5 and 8.5 Å, and applied on a group-by-group basis to avoid artificially splitting dipoles. The above cutoff has been employed to minimize the computational cost and accomplish large scale simulations of this magnitude. With this approach, the protein is well behaved, however, highly charged residues are susceptible to inaccuracies.

The simulations used the SPC model of water and employed periodic boundary conditions. The protocol for simulation at 300 K included a 500-step minimization using the conjugate gradient method, a heating step for 1.5 ps, wherein the temperature of the entire system was raised to 300 K, a Gaussian equilibration for 3.5 ps, an unconstrained MD equilibration for 15.0 ps followed by the trajectory. The simulation at 600 K was similar to the 300 K simulation, with minor differences that include a 3.0-ps heating step and a 7.0-ps Gaussian equilibration. The temperature window was 5 K during the equilibration stages, and 10 K during the trajectory for all simulations. The total length of the simulations are 180 ps at 300 K and 200 ps at 600 K. The calculations were carried out on Cray Y-MP machines at the Pittsburgh Supercomputing Center and at the National Cancer Institute in Frederick, MD.

Secondary structural regions from the MD trajectory were individually superposed on the corresponding region in the crystal structure to derive the root mean square deviation (RMSD) for that region. The deviations from all secondary structures were then segregated into helical and sheet regions and the average RMSD per atom is plotted as a function of time. Only backbone atoms were used in the analysis. Conformational analysis was carried out by the Dials and Windows program (Ravishanker et al., 1989) in the MD Toolchest (Ravishanker and Beveridge, 1993). RMS fluctuations of ϕ and ψ angles with respect to those found in the crystal form were independently determined and averaged over both angles and over all residues present within a given secondary structural motif. The extent of secondary structure was calculated from the ϕ and ψ angles, based on the method of Daggett and Levitt (Daggett and Levitt, 1992b). The mean value and spread of ϕ and ψ angles were first determined from the crystal structure for the helical and sheet regions, respectively. The trajectory and reference structure were then analyzed based on whether the ϕ and ψ angles for each residue was within the dispersion observed in the crystal structure, separately for helices and sheets.

The extent of secondary structure in the protein was also determined based on a combined analysis of backbone hydrogen bonds and [ϕ, ψ] angles. Essentially, the analysis involves three steps: (*a*) identifying all backbone hydrogen bonds using a distance and angle criteria and filtering out all weak hydrogen bonds based on a crude energy calculation; (*b*) assigning residues to secondary structural types using the criteria of Kabsch and Sander (1983) based on the hydrogen bonds identified in the previous step, and (*c*) filtering out residues whose [ϕ, ψ] values differ by more than 50° of their ideal values (Daggett and Levitt, 1992a). The number of residues within a given secondary structural type is then summed and expressed as a percentage with respect to the corresponding data for the crystal form. This method (Vijayakumar et al., 1994) has the advantage of considering the backbone structure at both spatially and sequentially neighboring residues for a proper evaluation and/or assignment of secondary structural regions.

The criteria used for identifying the backbone hydrogen bonds are as follows. We used an H—H ··· O=C distance cutoff of 2.25 ± 0.75 Å, an N—H ··· O angle cutoff of 150 ± 30°, and an energy filter which discards any hydrogen bond weaker than −1.0 kcal/mol. The hydrogen bond energy was assumed to arise only from the electrostatic interaction between the atoms involved in the hydrogen bond, namely, N, H, C, and O, and calculated based on the partial atomic charges used in the GROMOS force field. While assigning residues to secondary structures we have used the following priority for overlapping regions: α-helices > β-sheets > 3_{10}-helices. However, note that the data reported for helices includes both, those of α- and 3_{10}-helices.

RESULTS

The convergence criteria for the simulation of the wild type enzyme in solution at 600 K are shown in Fig. 2. The total energy (Fig. 2 *a*) can be seen to reflect through a series of velocity rescalings during equilibration, but remains constant after 81 ps. A major rescaling occurs at about 80 ps and precedes the onset of the differential unfolding. The rescaling points in the total energy are accompanied by corresponding changes in the potential energy (Fig. 2 *b*). The temperature of the system (Fig. 2 *c*) remains steady and fluctuates at 600 ± 5 K. The RMS deviation of the backbone atoms (Fig. 2 *d*) increases steadily to about 6.5 Å by 170 ps and then reaches a plateau during the last 30.0 ps. In comparison, RMSD of the backbone atoms in the room temperature simulation leveled off around 1.8 Å. Collectively these criteria indicate that the high temperature simulation has found a relatively stable potential energy region in the unfolding pathway, which may be provisionally associated with an intermediate state. Thus, it appears to be a reasonable point in the unfolding pathway to stop the simulation for further analysis.

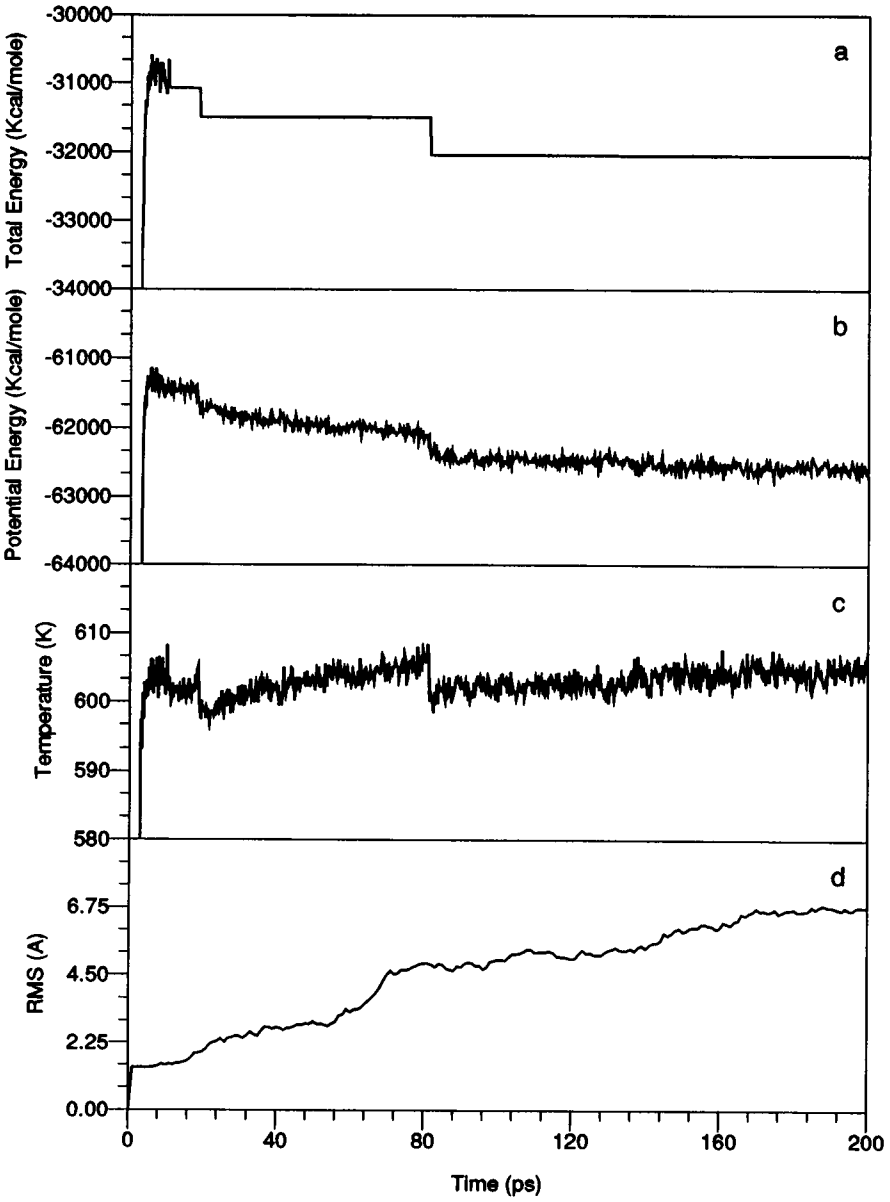

FIGURE 2 Convergence criteria for the simulation at 600 K. (*a*) Total energy; (*b*) potential energy; (*c*) temperature; and (*d*) RMS deviation for backbone atoms plotted as a function of time.

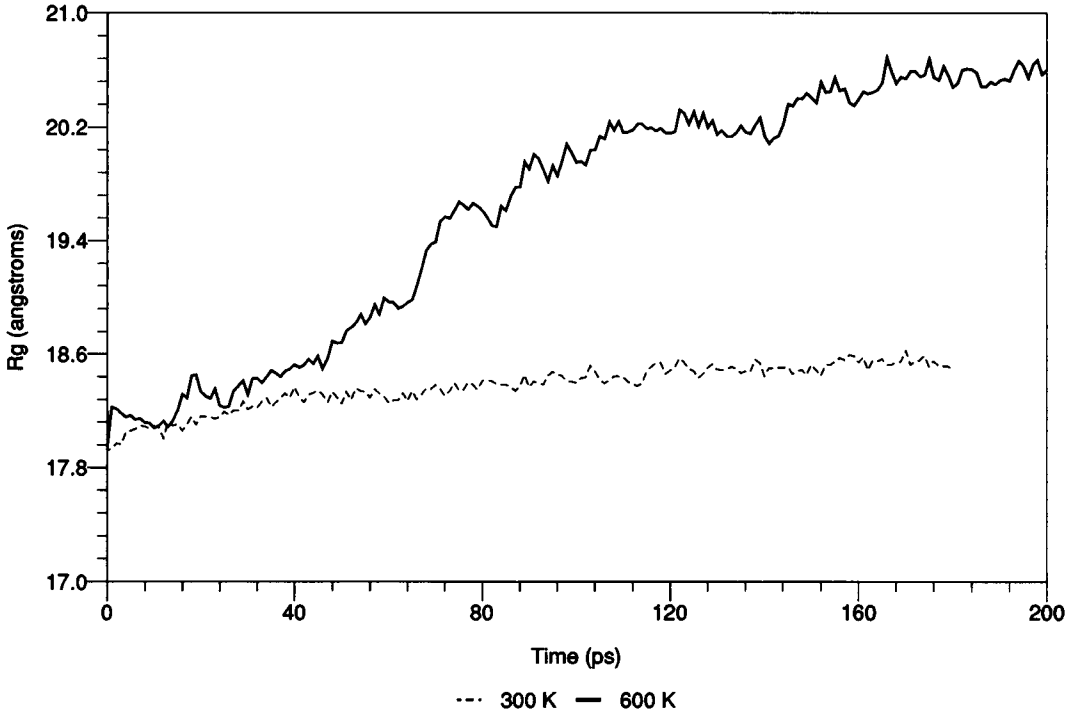

FIGURE 3 Comparison of the radius of gyration between the simulations at 300 and 600 K. The Rg for the crystal structure is 17.92 Å.

The radius of gyration (Rg) was computed along the trajectory of the 600 K simulation and compared with that of a corresponding room temperature simulation (Fig. 3). Both simulations indicate that the enzyme undergoes expansion with respect to the crystal form (Rg = 17.92 Å), but differ in their extent and persistence. The simulation at room temperature shows very nominal expansion, consistent with the relaxation of the protein in the solvent environment. The 600 K simulation can be divided into four phases based on Rg. In phase I (0–40 ps), we see an nominal increase in Rg similar to that in the room temperature simulation, but slightly larger owing to higher temperature. Phase II (40–105 ps), is marked by a rapid expansion and onset of thermal unfolding. In phase III (105–145 ps), the expansion levels off and Rg is stable for about 40 ps, while the molecule continues to denature differentially among helices and sheets. Phase IV (145–200 ps), remains slightly more expanded than the third and denaturation continues further. Expansion of the enzyme in the simulation at high temperature is significant (about 16%) and, the expansion is sustained.

To observe the morphological changes in the molecule, the MD coordinates were used to generate a graphics animation. Six representative snapshots from the MD trajectory are shown in Fig. 4. At the outset, the overall shape of the molecule appears to be retained throughout. The structure at 0 ps (*top left*) is shown as a reference. The structure at 30 ps (*top center*) represents the first phase of unfolding. The enzyme is intact during this phase but begins to expand rapidly soon after as illustrated by structures at 60 ps (*top right*) and 70 ps (*bottom left*). The structure at 115 ps (*bottom center*) is representative of the third phase of unfolding, where itremains expanded and the phenomenon of differential stability is manifest. Most of the helices appear to have melted, at least partially, while the five-stranded anti-parallel β-sheet is nearly intact, with the exception of the edge strand that is proximal to the amino- and carboxyl-terminal helices. The structure at 200 ps (*bottom right*) is representative of the fourth phase and marks the end of the unfolding simulation. This phase is slightly more expanded than the third and shows further break down of both helices and sheets. It is

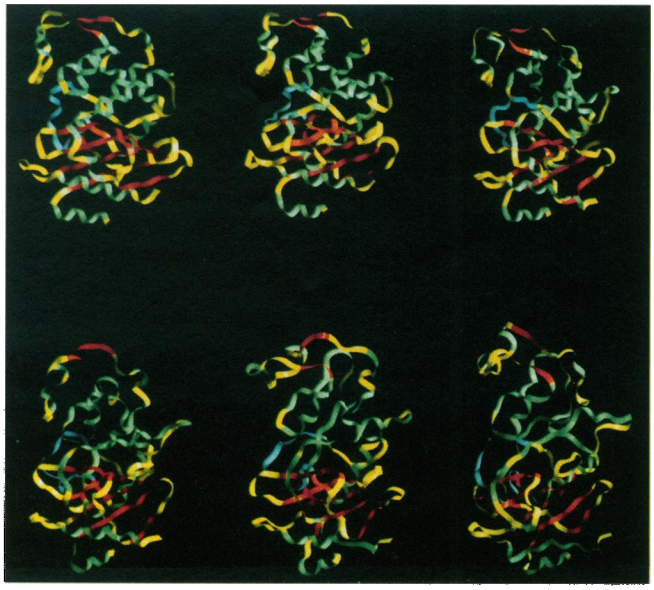

FIGURE 4 Snapshots from the MD simulation at 600 K. Structures from left to right and top to bottom are at 0, 30, 60, 70, 115, and 200 ps. The pictures are color coded the same as in Fig. 1.

noteworthy that the amino-terminal helix (which is fully solvent exposed) is fairly stable even at the end of the simulation, but the carboxyl-terminal helix (also solvent exposed, but to a lesser extent) has almost completely melted.

The RMSD of the backbone atoms were computed separately for helical and sheet regions as described under Methods. The deviations for the 600 K simulation, shown in Fig. 5 for both regions, steadily increase during phase I and II. With the onset of phase III, the RMSD for helices continue to rise while that for sheets levels off and remains so till the end of this phase. During the early part of phase IV, there is a significant increase in the RMSD for sheets which brings it closer to that of helices, however, it begins to level off during the latter half and remains appreciably lower than that of helices. This tends to suggest that, perhaps, the unfolding in the case of sheets proceeds in short bursts. Nonetheless, these results suggest a differential stability for sheets and helices during unfolding with the melting of helical motifs preceding that of sheets. Significant difference in the RMSD between helical and sheet regions are not seen in the control simulations at room temperature. It is thus evident that the observed effects arise mainly from thermal denaturation. The removal of amino- and carboxyl-terminal helices from the computation of RMS did not have much influence on the deviation observed for α-helices.

Conformational analysis of the trajectory was carried out to study the variation in dihedral angles ϕ, ψ, and ω. All peptide bonds remained *trans* with the exception of the bond between Glu^{166} and Ile^{167}, which is *cis* in the starting structure and remained so during the simulation. The RMS fluctuation of ϕ and ψ angles averaged over both angles and all residues within the helical and sheet regions, respectively, is presented in Fig. 6 for both simulations. At room temperature the backbone dihedral angles fluctuate to a greater extent for sheets than for helices. However, at high temperature this behavior is retained only during the initial stages (phase I and part of phase II). As the enzyme begins to expand the fluctuation in the helical region begins to increase dramatically over that of β-sheets, and a substantial difference is retained till the end of the simulation. Toward the end of phase III, the fluctuation in the sheet region increases slightly and appears to level off during phase IV. Overall, this analysis suggests that the helical regions are less flexible than sheets at room temperature, while at high temperature their relative flexibility is reversed.

The backbone dihedral angles were further analyzed to determine the extent of secondary structure remaining within the helical and sheet regions, respectively. The time evolution of the percentage (w.r.t. crystal structure) helical and sheet structure is presented in Fig. 7, for both simulations. The criteria used for this analysis was based on the dispersion found in the crystal structure and are as follows: for helices we employed a range of $-88 < \phi < -40$ and $-66 < \psi < -11$ and for sheets the range included $-166 < \phi < -78$ and $98 < \psi < 173$. From Fig. 7, it is evident that the number of residues in the helical region drops off quite rapidly in the 600 K simulation. On the contrary, the number of residues in the sheet region is greater than in the crystal structure, at the end of the simulation. Note however, there is no significant difference between the helical and sheet regions for the simulation at 300 K, although there is a small decrease in their secondary structural content.

The time evolution of the extent of secondary structure retained during the MD trajectory was also determined based on a combined analysis of backbone hydrogen bonds and $[\phi, \psi]$ angles, as described under Methods. The results averaged over all helical and sheet regions, respectively, are

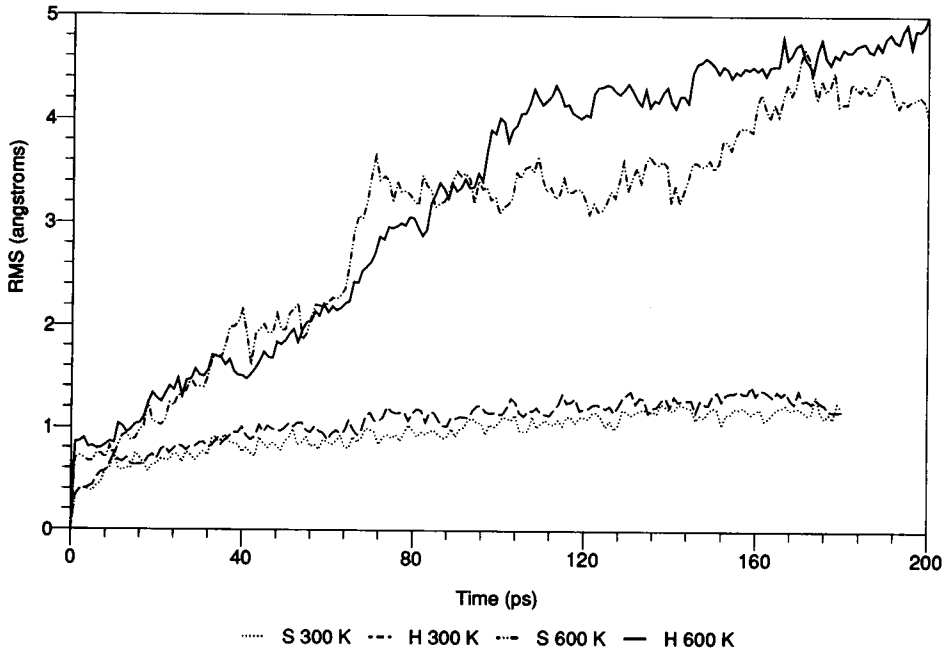

FIGURE 5 RMS deviation of backbone atoms in cartesian space, averaged over all residues in the helical and sheet regions, respectively, shown as a function of time for the simulations at 300 and 600 K. The deviations were calculated one secondary structure at a time by superposing only the backbone atoms within each secondary structure over the corresponding atoms in the crystal structure.

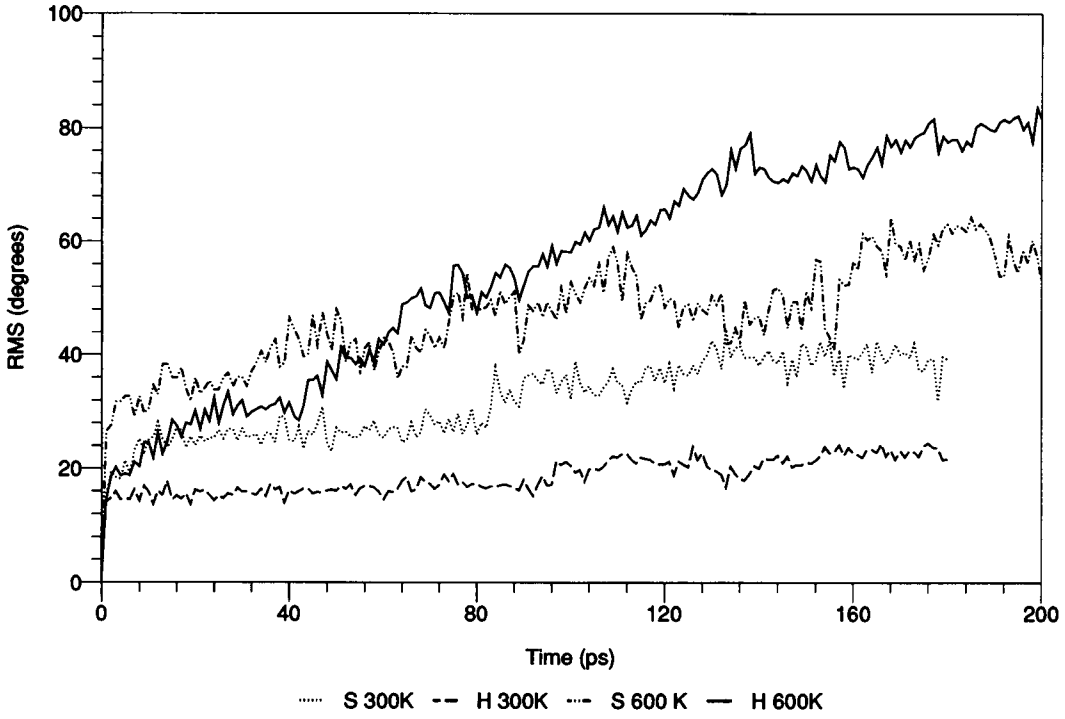

FIGURE 6 Time evolution of the RMS fluctuations in [ϕ, ψ] angles averaged over both angles and all residues in the helical and sheet regions, respectively, shown for the simulations at 300 and 600 K.

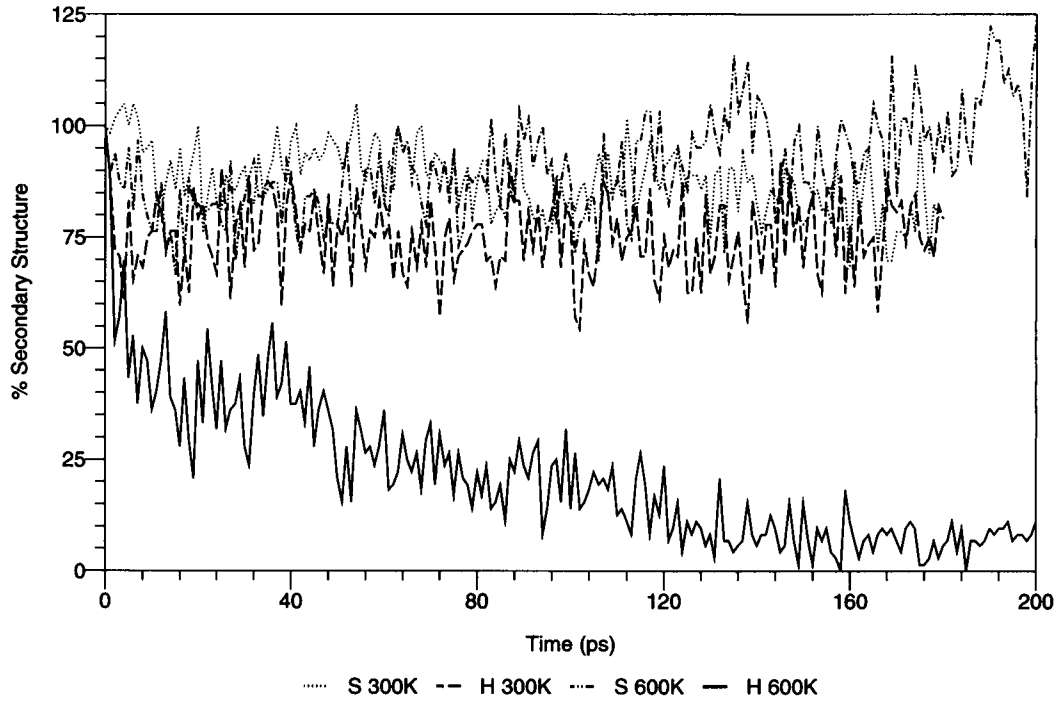

FIGURE 7 Time evolution of the percentage secondary structure determined from analysis of [ϕ, ψ] angles, present within the helical and sheet regions, respectively. Percentages reported are with respect to corresponding data for the crystal structure. The criteria used for determining the helical and sheet residues are described in the text.

plotted in Fig. 8. This analysis allows us to differentiate the melting property of secondary structural components of a protein dynamically. In the simulation at 300 K, the helical structures were retained around 100% (i.e., the value found in the crystal structure). However, note that there is a small increase in the extent of sheet structure. Differential unfold-

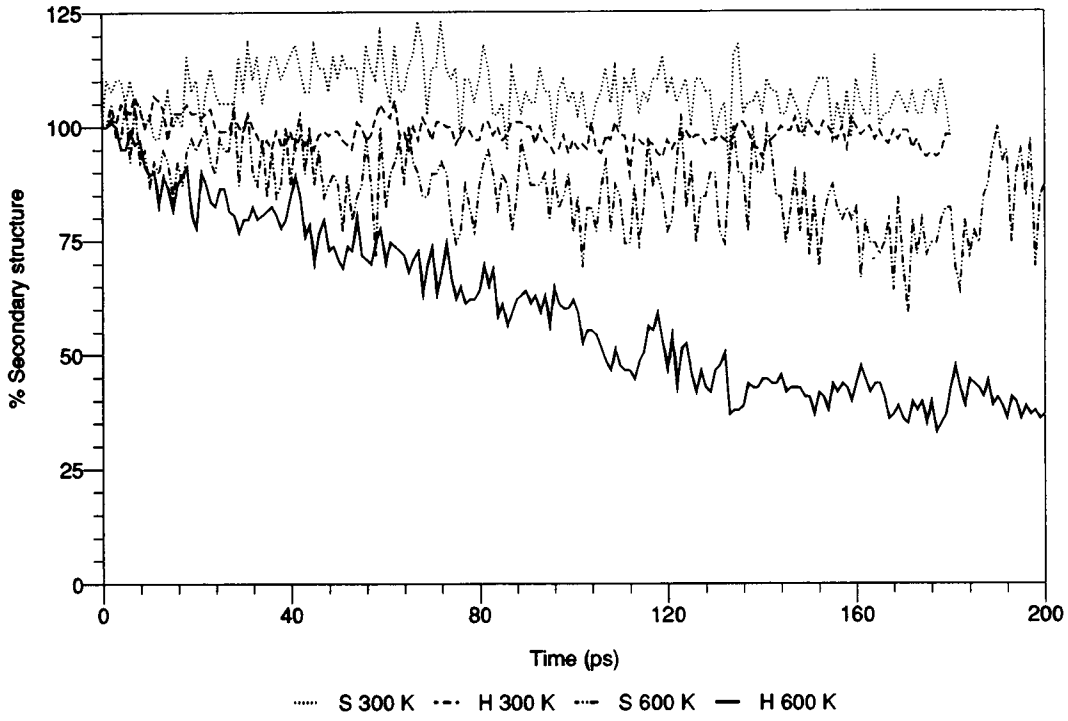

FIGURE 8 Time evolution of the percentage secondary structure determined from analysis of backbone hydrogen bonds and [ϕ, ψ] angles, as described under Methods. Percentages reported are with respect to corresponding data for the crystal structure.

ing can be seen in the simulation at 600 K. Approximately 85–90% of the sheet structure is retained at the end of the simulation, while only 35–40% of the helical structures are retained. This compares with an experimental value of 50% deformation for helices and no apparent change for sheets in state H of β-lactamase (Carrey and Pain, 1978). It is worthy to note that the hydrogen bond analysis alone does not bring out the differential stability phenomenon. However, it does support other observations in a qualitative manner. Also note that there is a significant increase in $i/i+2$ hydrogen bonds in the simulation at 600 K, similar to the trend observed in the high temperature simulation of lysozyme (Mark and van Gunsteren, 1992).

DISCUSSION

The simulation and analysis of the dynamical motions of the thermal unfolding of β-lactamase at 600 K reveals several notable phenomena. The preferential thermal stability of β-sheet motifs over α-helices clearly emerges from the relatively lower RMS deviation for β-sheets both in cartesian and [ϕ, ψ] space and the greater percentage of sheet residues retained than those of helices (Figs. 7 and 8). The radius of gyration and the graphics animation of MD coordinates shows that the molecule is slightly expanded compared to the room temperature simulation, and retains its globular shape. The expansion is significant (about 16%) and is sustained. Experimental studies indicate state H to be considerably expanded ($r/r_0 \sim 1.7$), whereas state I is only slightly larger compared to the native enzyme (Carrey and Pain, 1978; Ptitsyn et al., 1990). Folded states of mutants P2 and P54, which have been shown to be similar to state I of native enzyme, show an increase in Stokes' radius of only 5–12%. Gel exclusion chromatography on β-lactamase 1 from *Bacillus cereus* has yielded a Stokes' radius for the A state of β-lactamase that is 10–20% larger than that of the native enzyme. All of the above data are estimates based on a spherical model for the enzyme and thus they are only qualitatively meaningful. Putative unfolding intermediates accessed by the present high temperature study are thus consistent with experimentally observed folding intermediates, particularly state I.

The onset of differential stability of β-sheets and α-helices (Figs. 5 and 6) is observed about 100 ps into the simulation and persists for the duration of the simulation. Once the sheet structure is weakened and reaches an arbitrary threshold, the differential stability begins to diminish. The higher intrinsic stability of sheets observed in β-lactamase may be attributed in part to the cooperative effect of hydrogen bonds and other noncovalent interactions distributed over a large number of residues. In addition, conformational entropy may also be operating since the backbone [ϕ, ψ] angles can enjoy a greater degree of freedom without destabilizing the sheet structure. The latter effect is intuitively obvious from the Ramachandran map (Ramachandran et al., 1963) which indicates a large conformational space available to sheets when compared to helices. This also accounts for the greater fluctuation of [ϕ, ψ] angles at 300 K in sheet motifs compared to that of helices.

The extent to which the differential stability of β-sheets is case specific to β-lactamase is yet to be established. Daggett and Levitt have examined the intrinsic stability of helices and

sheets in BPTI at room temperature and high temperatures and at different oxidation states (Daggett and Levitt, 1992a). The stability of protein secondary structures in aqueous solution have been studied by the application of MD to the folding/unfolding process in model peptides (Tobias et al., 1991). Free energy surfaces derived from these simulations indicate the β-sheet motif to be considerably more stable than the others. These authors have suggested that a difference in the intrinsic stability of secondary structural motifs could play a role in their formation and consequently in protein folding. If we assume that the intermediates observed in our unfolding studies are also intermediates in the folding process, our results suggest that β-sheets may be formed early on in the folding pathway and provide a stable scaffolding for the rest of the molecule to fold. Recent NMR studies on the folding mechanism in RNase A (Udgaonkar and Baldwin, 1990, 1988) using pulsed proton exchange support our above hypothesis. The results show one principal early forming intermediate in which probes that are hydrogen-bonded within the β-sheet region are protected to a greater extent than in the helical region and the protection factor was observed to increase with time from the start of folding. NMR studies have also been done on α-lactalbumin and cytochrome *c*, but the most protected amide protons in these systems occur mainly in the helical regions. It must be pointed out that both α-lactalbumin and cytochrome *c* are predominantly helical in nature. While α-lactalbumin contains only two β-strands, each spanning three to four residues, cytochrome *c* has none. If some local secondary structures (helices, sheets, loops, and turns) are formed soon after or during protein transcription, these local secondary structures in combination with hydrophobic interactions could drive the structure to compactness. At this point a scaffolding is formed which brings together sequentially distant regions to close proximity and puts the most stable elements in place, leading to overall shape and globularity. The final tertiary structure is then obtained by fine tuning of the main chain and side chain interactions. In the case of α-proteins, this scaffolding can be obtained by mutual stabilization of the helices owing to dipolar interactions. Evidence supporting this idea has been observed by proton NMR in the folding intermediates of cytochrome *c*.

The high temperature MD-simulated structures of β-lactamase can be characterized as globular structures with morphology similar to the x-ray structure. Complete and partial secondary structures are persistent in these structures. The loop regions start fluctuating first when all secondary structures are still intact. After that, the secondary structures begin to deform with the melting of helical motifs preceding that of sheets. These results show that the structures obtained during 600 K simulation have the characteristics of a molten globule. Details of individual secondary structures and characteristic features of the molten globule state will be published elsewhere. It will be interesting to see if the simulated structures can be identified with the experimentally observed intermediates, states H and I (Goto and Fink, 1989; Thomas et al., 1983), by future experiments.

The present studies suggest a preferential stability for β-sheets over α-helices based on a computer simulation of the thermal unfolding of β-lactamase in solution. A model for protein folding consistent with the above hypothesis and recent experimental data on the folding intermediates of a predominantly β-protein, RNase, has been proposed. It must be emphasized that the preferential stability of β-sheets need not be absolute and could vary depending on the local environment and experimental conditions. Nevertheless, it has been proposed as a possible general feature in β and α/β proteins and remains to be further validated. Our comparisons to experiment is only qualitative since data on intermediate structures are difficult to obtain and are limited to a few small systems that have been extensively characterized. However, with rapidly emerging technology more detailed studies should be possible on much larger systems, in the near future. Our results demonstrate the usefulness of computer simulations in furthering our understanding of biochemical processes, which are in many ways complementary to experimental methods.

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REFERENCES

Branden, C., and J. Tooze. 1991. Introduction to Protein Structure. Garlan, New York.

Carrey, E. A., and R. H. Pain. 1978. Conformation of a stable intermediate on the folding pathway of Staphylococcus aureus penicillinase. *Biochim. Biophys. Acta* 533:12–22.

Christensen, H., and R. H. Pain. 1991. Molten globule intermediates and protein folding. *Eur. Biophys. J.* 19:221–229.

Craig, S., N. Hollecker, T. E. Creighton, and R. H. Pain. 1985. Single amino acid mutations block a late step in the folding of β-lactamase from Staphylococcus aureus. *J. Mol. Biol.* 185:681–687.

Creighton, T. E., and R. H. Pain. 1980. Unfolding and refolding of Staphylococcus aureus penicillinase by urea gradient electrophoresis. *J. Mol. Biol.* 137:431–436.

Daggett, V., and M. Levitt. 1992A. A Model of the molten globule state from molecular dynamics simulations. *Proc. Natl. Acad. Sci. USA* 89:5142–5146.

Daggett, V., and M. Levitt. 1992B. Molecular dynamics simulations of helix denaturation. *J. Mol. Biol.* 223:1121–1138.

Dobson, C. M. 1991. Characterization of protein folding intermediates. *Curr. Opin. Struct. Biol.* 1:22–27.

Englander, W. S., and L. Mayne. 1992. Protein folding studied using hydrogen-exchange labeling and two-dimensional NMR. *Annu. Rev. Biophys. Biomol. Struct.* 21:243–265.

Fan, P., D. Kominos, D. B. Kitchen, R. M. Levy, and J. Baum. 1991. Stabilization of α-helical secondary structure during high temperature molecular dynamics of α-lactalbumin. *J. Chem. Phys.* 158:295–301.

Goto, Y., and A. L. Fink. 1989. Conformational states of β-lactamase: molten-globule state at acidic and alkaline pH with high salt. *Biochemistry* 28:945–952.

Goto, Y., L. J. Calciano, and A. L. Fink. 1990a. Acid-induced folding of proteins. *Proc. Natl. Acad. Sci. USA.* 87:573–577.

Goto, Y., N. Takahashi, and A. L. Fink. 1990b. Mechanism of acid-induced folding of proteins. *Biochemistry* 29:3480–3488.

Herzberg, O. 1991. Refined crystal structure of β-lactamase from Staphylococcus aureus PC1 at 2.0 Å resolution. *J. Mol. Biol.* 217:701–719.

Kabsch, W., and C. Sander, 1983. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577–2637.

Kim, P. S., and R. L. Baldwin. 1990. Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* 59:631–660.

Mark, A. E., and W. F. van Gunsteren. 1992. Simulation of the thermal denaturation of hen egg white lysozyme: trapping the molten globule state. *Biochemistry* 31:7745–7748.

Matouschek, A., J. T. Kellis, Jr., L. Serrano, M. Bycrofft, and A. R. Fersht. 1990. Transient folding intermediates characterized by protein engineering. *Nature (Lond)* 346:440–445.

Ptitsyn, O. B., R. H. Pain, G. V. Semisotnov, E. Zerovnik, and O. I. Razgulyaev. 1990. Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* 262:20–24.

Ramachandran, G. N., C. Ramakrishnan, and V. Sasisekharan. 1963. Stereochemistry of polypeptide chain configurations. *J. Mol. Biol.* 7:95–99.

Ravishanker, G., and D. L. Beveridge. 1993. MD Toolchest 2.0. Wesleyan University, Middletown, CT 06459.

Ravishanker, G., S. Swaminathan, and D. L. Beveridge. WESDYN 2.0. 1993. Wesleyan University, Middletown, CT 06459.

Ravishanker, G., S. Swaminathan, D. L. Beveridge, R. Lavery, and H. Sklenar. 1989. Conformational and Helicoidal Analysis of 30 psec of Molecular Dynamics on the d(CGCGAATTCGCG) Double Helix. *J. Biomol. Struct. Dyn.* 6:669–699.

Roder, H., G. A. Elove, and S. W. Englander. 1988. Structural characterization of folding intermediates in cytochrome c by H-exchange labelling and proton NMR. *Nature* 335:700–704.

Swaminathan, S. WESDYN 1.0. 1990. Wesleyan University, Middletown, CT 06459.

Thomas, R. M., J. Feeney, R. B. Nicholson, R. H. Pain, and G. C. K. Roberts. 1983. Identification by NMR spectroscopy of a stable intermediate structure in the unfolding of Staphylococcal β-lactamase. *Biochem. J.* 215: 525–529.

Tirado-Rives, J., and W. L. Jorgensen. 1993. Molecular dynamics simulations of the unfolding of apomyoglobin in water. *Biochemistry* 32:4175–4184.

Tobias, D. J., S. F. Sneddon, and C. L. Brooks. 1991. The stability of protein secondary structures in aqueous solution. In *Advances in Biomolecular Simulations*, AIP Conf. Proc. 239:174–199.

Udgaonkar, J. B., and R. L. Baldwin. 1988. NMR evidence for an early frame work intermediate on the folding pathway of ribonuclease A. *Nature* 335:694–699.

Udgaonkar, J. B., and R. L. Baldwin. 1990. Early folding intermediate of ribonuclease A. *Proc. Natl. Acad. Sci. USA* 87:8197–8201.

van Gunsteren, W. F., and H. J. C. Berendsen. GROMOS86: Groningen Molecular Simulation System. 1986. University of Groningen, Groningen.

Vijayakumar, S., S. Vishveshwara, G. Ravishanker, and D. L. Beveridge. 1994. A method to analyze the intrinsic stability of protein secondary structures in molecular dynamics simulation. In *Modeling the Hydrogen Bond*. Smith, D. A., editor. ACS Symposium series. In press.