Structural and Dynamic Effects of α-Helix Deletion in Sso7d: Implications for Protein Thermal Stability

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ABSTRACT **Sso7d is a 62-residue protein from the hyperthemophilic archaeon *Sulfolobus solfataricus* with a denaturation temperature close to 100°C around neutral pH. An engineered form of Sso7d truncated at leucine 54 (L54Δ) is significantly less stable, with a denaturation temperature of 53°C. Molecular dynamics (MD) studies of Sso7d and its truncated form at two different temperatures have been performed. The results of the MD simulations at 300 K indicate that: (1) the flexibility of Sso7d chain at 300 K agrees with that detected from X-ray and NMR structural studies; (2) L54Δ remains stable in the native folded conformation and possesses an overall dynamic behavior similar to that of the parent protein. MD simulations performed at 500 K, 10 ns long, indicate that, while Sso7d is in-silico resistant to high temperature, the truncated variant partially unfolds, revealing the early phases of the thermal unfolding pathway of the protein. Analysis of the trajectories of L54Δ suggests that the unzipping of the N-terminal and C-terminal β-strands should be the first event of the unfolding pathway, and points out the regions more resistant to thermal unfolding. These findings allow one to understand the role played by specific interactions connecting the two ends of the chain for the high thermal stability of Sso7d, and support recent hypotheses on its folding mechanism emerged from site-directed mutagenesis studies. Proteins 2004; 57:692–701.** © 2004 Wiley-Liss, Inc.

Key words: hyperthermophilic protein; thermal stability; molecular dynamics; essential dynamics; unfolding pathway; Sso7d

INTRODUCTION

Sso7d is a small, 62-residue protein,[1] from the hyperthermophilic archaeon *Sulfolobus solfataricus*.[2] Sso7d is very rich in lysines and lacks in hystidines, asparagines and cysteines. It has high thermal stability, with a denaturation temperature of 98°C around neutral pH, and is stable in very acid solutions.[3] Sso7d shows several biological activities: (1) it is able to bind DNA and has been suggested to have a role in DNA packing and protection at high temperature;[4,5] (2) it has a ribonuclease activity, even though the residues constituting its active site were not clearly identified[6–8]; and (3) it has a chaperon-like function.[9]

The three-dimensional solution structure of Sso7d was determined by means of NMR spectroscopy[4,8] (PDB codes 1SSO and 1JIC) (Fig. 1). It consists of an incomplete β-barrel built up of a double-stranded β-sheet (residues 2–7 and 10–15) packed against a triple-stranded β-sheet (residues 21–25, 28–33, and 41–46), which ends at the C-terminus with an α-helix (residues 47–61). The structures of several complexes of Sso7d with DNA have also been solved by both X-ray crystallography[10–12] and NMR.[13] These structures have shown that the protein preserves its overall conformation upon DNA binding, and that the residues involved in the protein-DNA interactions are located on the surface of the triple stranded β-sheet.

Recently, a truncated variant of Sso7d, lacking 8 residues at the C-terminus, called L54Δ, has been produced.[14] It has been shown that L54Δ has a folded structure around neutral pH with a denaturation temperature of 53°C, 45°C lower than that of Sso7d, and is able to bind DNA.[14] Since the molecular bases of the different thermal stability between Sso7d and L54Δ were not fully understood,[14] we decided to study the structural and dynamic effects of the α-helix truncation by means of molecular dynamics (MD) simulations.

This technique is a powerful tool for investigating the structural and dynamic behavior of biological macromolecules.[15,16] MD simulations have been frequently used to study the effect of single point mutations[17,18] as well as the stability of protein fragments.[19] A number of computational studies have also started addressing features of protein thermal stability.[20–31] In the present case, molecular dynamics/essential dynamics studies of Sso7d and L54Δ were performed in water at two different temperatures.

Abbreviations: Sso7d, 7 kDa DNA-binding protein from *Sulfolobus solfataricus;* L54Δ, variant form of Sso7d truncated at leucine 54; MD, molecular dynamics; ED, essential dynamics; RMSD, root mean square deviations; RMSF, root mean square fluctuations; RMSIP, root mean square inner product.

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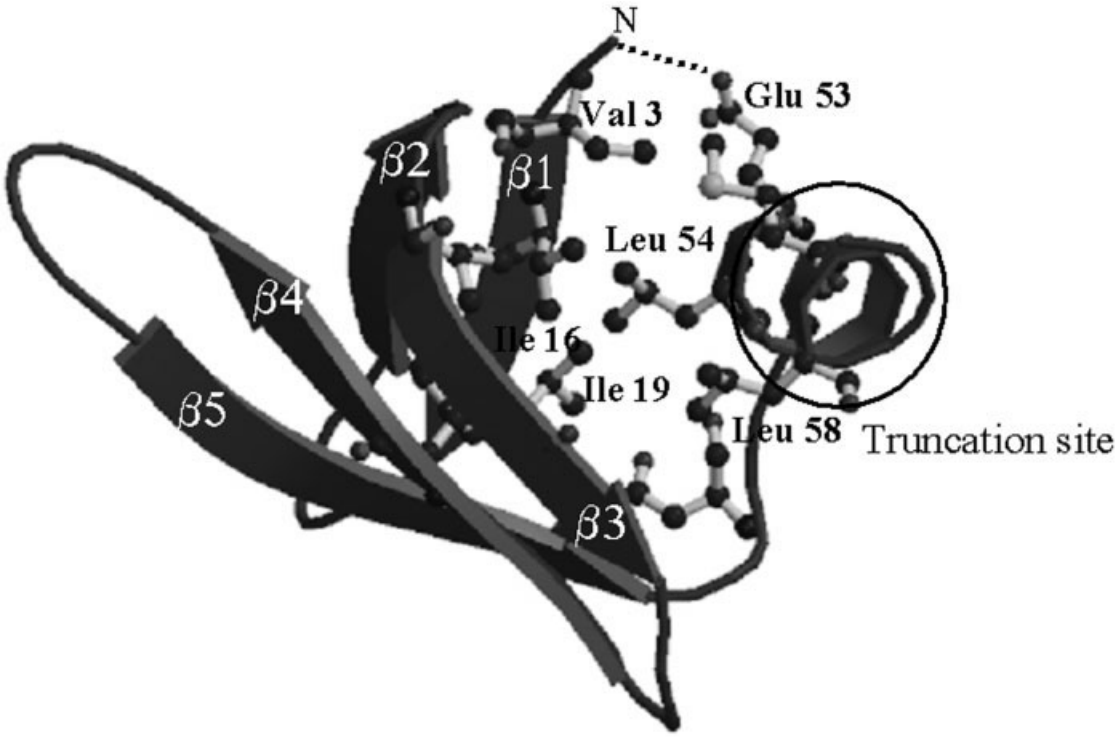

Fig. 1. Ribbon diagram of the NMR structure of Sso7d.

The results show that L54Δ has a folded structure at 300 K, close to that of the corresponding part of Sso7d, in agreement with the experimental findings.[14] Essential dynamics analysis reveals that at room temperature Sso7d and L54Δ exhibit similar overall dynamic motions. The results of the MD simulations performed at 500 K show that, while Sso7d remains stable, the truncated variant partially unfolds. Analysis of the trajectories of L54Δ suggests that the breaking of interactions connecting residues located at the chain termini plays a crucial role in the unfolding pathway. Moreover, the central strands β3 and β4 appear very resistant to thermal unfolding, and should represent the folding nucleus of Sso7d, in line with experimental evidence.[32]

METHODS

The starting structure for the MD simulations of Sso7d was the average NMR structure determined by Consonni and co-workers[8,33] taken from the Protein Data Bank file 1JIC (Fig. 1). The starting structure for the MD simulations of L54Δ was modeled by deleting the last 8 residues from the C-terminal region of Sso7d. The two models were immersed in cubic boxes ($7 \times 7 \times 7$ nm^3) filled with simple-point-charge water molecules.[34] The resulting system was first energy minimized. The minimization was followed by an MD equilibration stage of 20 ps during which the protein atoms were restrained to their starting positions. The systems were finally re-minimized before starting MD runs.

All MD simulations were performed with the GROMACS program,[35] using a procedure described elsewhere.[36,37] Briefly, a cut-off of 14 Å was used for the treatment of both the electrostatic and the Lennard-Jones interactions. All bond lengths were constrained by Lincs.[38] The Newton's equations of motion were integrated with a time step of 2 fs. Atomic coordinates were saved for analyses every 0.5 ps. A dielectric constant of 1 was used. The temperature was kept constant by coupling the system with an external bath.[39] The overall charge of the systems was neutralized by adding the appropriate number of chloride ions more than 7 Å away from the protein surface.

MD simulations were produced for each system using an effective water density of 0.997 g cm^{-3} at both 300 and 500 K. In the last case, two simulations were performed. In order to reduce the effect of the excess pressure, four additional MD simulations at 500 K on L54Δ were performed using a reduced water density, 0.854 g cm^{-3}, as suggested by Daggett and co-workers.[27,28] This density value, obtained by expanding the box volume, corresponds to the equilibrium vapor pressure of liquid water at 500 K.[27,28]

Essential degrees of freedom were extracted from the equilibrated portions of the trajectories according to the essential dynamics (ED) method,[40] based on the diagonalization of the covariance matrix of the coordinate fluctuations. The obtained eigenvectors represent the directions of motion, and the eigenvalues the amount of motion along each eigenvector. The eigenvectors are sorted in descending order according to their eigenvalues. Usually, the first 10 eigenvectors are used to describe almost all the confor-

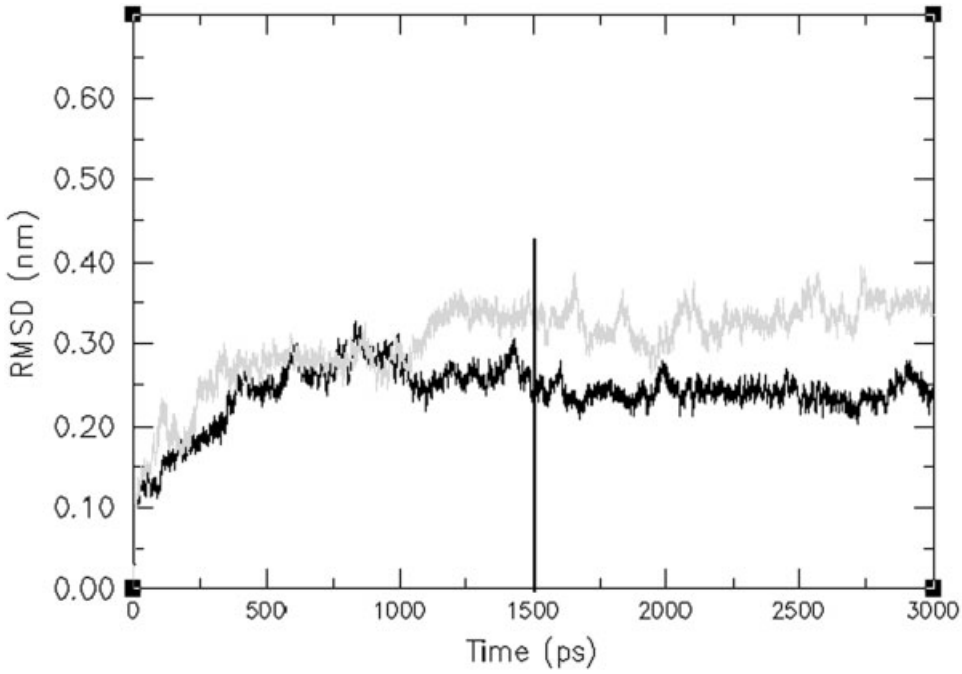

Fig. 2. Time course of the root mean square positional deviations from the NMR starting structure computed on C^{α} atoms for Sso7d (black line) and L54Δ (gray line) in the 300-K simulations.

mational sub-states accessible to the protein (essential subspace).

Only C^{α} atoms were included in the definition of the covariance matrices. The convergence in the essential subspace (first 10 eigenvectors) was checked by using the root mean square inner product (RMSIP) between two halves of the equilibrated trajectory.[41,42] The RMSIP is defined as:

$$\sqrt{\frac{1}{10}\sum_{i=1}^{10}\sum_{j=1}^{10}(\eta_i\eta_j)^2}$$

where η_i and η_j are the ith and jth eigenvectors from the first and second half of the equilibrated trajectory, respectively. The RMSIP value has also been used to evaluate the overlap of the essential subspaces spanned by the two proteins. This procedure was already used to compare the dynamic properties of other protein systems,[42,43] and to compare the essential subspace spanned by simulated and experimental structures.[43–45]

Snapshots from the simulations were extracted and examined using VMD[46] and O.[47] Diagrams were drawn using MOLSCRIPT.[48] Secondary structure assignments were performed using DSSP.[49]

RESULTS

MD Simulations at 300 K

Structural features

Structural and dynamic effects caused by the deletion of 8 residues in the C-terminal α-helix of Sso7d have been investigated by 3-ns MD simulations of both Sso7d and L54Δ at 300 K. Several geometric parameters were analyzed to control the stability of the MD simulations. In Figure 2, the time evolutions of the C^{α} root mean square deviations (RMSD) of Sso7d and L54Δ versus their starting structures (see Methods) are plotted. As expected, Sso7d remains close to the starting conformation with a RMSD of 2.4 Å in the last 1.5 ns. Larger deviations (>3 Å) are observed in the loop 7–11 and in the C-terminal region, as found in the ensemble of NMR structures.[8] In fact, the radius of gyration, the content of secondary structure, and the total number of main chain-main chain hydrogen bonds do not change during the simulation, and their average values are close to those experimentally observed (Table I). Also the structure of L54Δ does not change significantly during the last 1.5-ns MD trajectory, showing a RMSD of 3.3 Å, constant values for the radius of gyration and for the number of the main chain-main chain hydrogen bonds (see Fig. 2 and Table I). Only marginal fluctuations in the secondary structure are observed. Taken together, these data demonstrate that Sso7d and L54Δ retain a folded structure throughout the simulations at 300 K.

On this basis, average structures for Sso7d and L54Δ were calculated from the simulations in order to perform a detailed comparison. L54Δ proves to be very close to the corresponding part of Sso7d, with an RMSD of 1.6 Å. The largest variations are located in the region of the loop 25–28 and of the turn 47–54. A visual comparison (see Fig. 3) shows the close similarity of the two structures, in particular of the three stranded β-sheet, whose RMSD is as low as 0.8 Å. These results agree with the experimental findings that L54Δ has a compact folded structure around

TABLE I. Geometric Parameters Obtained From MD Simulations at 300 and 500 K of the Two Protein Systems Investigated[†]

300 K	Sso7d	L54Δ
Simulation time (ns)	3	3
C^{α} root mean square deviation (Å)	2.40 ± 0.12	3.33 ± 0.21
C^{α} radius of gyration (Å)	10.49 ± 0.12 (10.92)	10.41 ± 0.12
Main chain–main chain H-bonds	30 ± 2 (29)	23 ± 2
β-sheet content (%)	44 ± 5 (48)[a]	52 ± 4 (53)[a]

500 K	Sso7d[b]		L54Δ[b]	
Simulation time (ns)	10	10	10	10
C^{α} root mean square deviation (Å)	4.88 ± 0.34	6.15 ± 0.32	6.06 ± 0.44	7.14 ± 0.20
C^{α} radius of gyration (Å)	10.25 ± 0.15	10.61 ± 0.15	9.63 ± 0.24	9.45 ± 0.09
Main chain–main chain H-bonds	25 ± 3	21 ± 3	15 ± 3	16 ± 3
β-sheet content (%)	35 ± 10	34 ± 6	18 ± 9	15 ± 8

[†]Experimental values are reported in parentheses.
[a]CD measurements from Shehi et al.[14]
[b]Results from two simulations are reported.

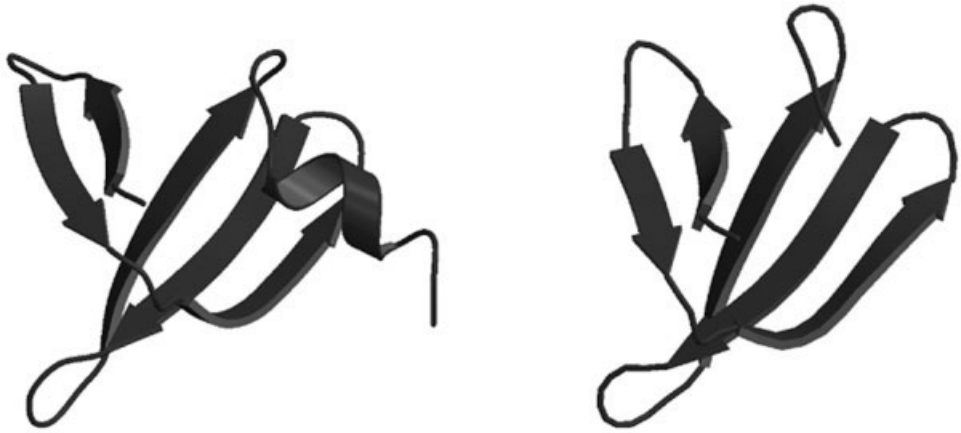

Fig. 3. Ribbon diagrams of the average MD structures of Sso7d and L54Δ derived from the 300-K simulations.

neutral pH, and is able to bind DNA as its parent protein.[14]

Dynamic features

Correlation matrices (data not shown) indicate that Sso7d and L54Δ have comparable concerted motions. Similar results are obtained by comparing the first few eigenvectors derived from the essential dynamics (data not shown). To compare quantitatively the essential subspace spanned by Sso7d and L54Δ, the RMSIP (see Methods for definition) between the first 10 eigenvectors of each simulation was calculated (Table II). The high value (0.70) of the RMSIP indicates that the essential subspace spanned by the two proteins is similar. These results demonstrate that Sso7d and L54Δ have a very similar global dynamic behavior, in line with our previous observation that proteins with analogous fold often exhibit similar concerted motions.[43]

The C^{α} root mean square fluctuations (RMSF), evaluated for each amino acid of Sso7d and L54Δ chains, indicate that the local flexibility is very similar in the two proteins (see Fig. 4). Loop regions show the higher mobility during simulations, in agreement with both NMR and X-ray experimental data.[8,11,12] Differences are only detected in the region 24–30, which is less flexible in L54Δ, and in the region of the truncation site, residues 48–54, which is more flexible in L54Δ.

TABLE II. Root Mean Square Inner Products for Projection of the First 10 (C^{α}) Eigenvectors of a Set Onto the First 10 of the Other

	Sso7d (300 K)	L54Δ (300 K)	Sso7d (500 K)	L54Δ (500 K)
Sso7d (300 K)	0.75[a]	0.70	0.66	—
L54Δ (300 K)		0.72[a]	—	0.53

[a]These values refer to the RMSIP obtained by comparing the two halves of the corresponding trajectory.

MD Simulations at 500 K

Sso7d does not unfold in MD simulations at 500 K

The time evolution of the structural and geometric parameters of Sso7d during the two 10-ns MD trajectories at 500 K shows that the protein is resistant in-silico to thermal unfolding over the investigated simulation time

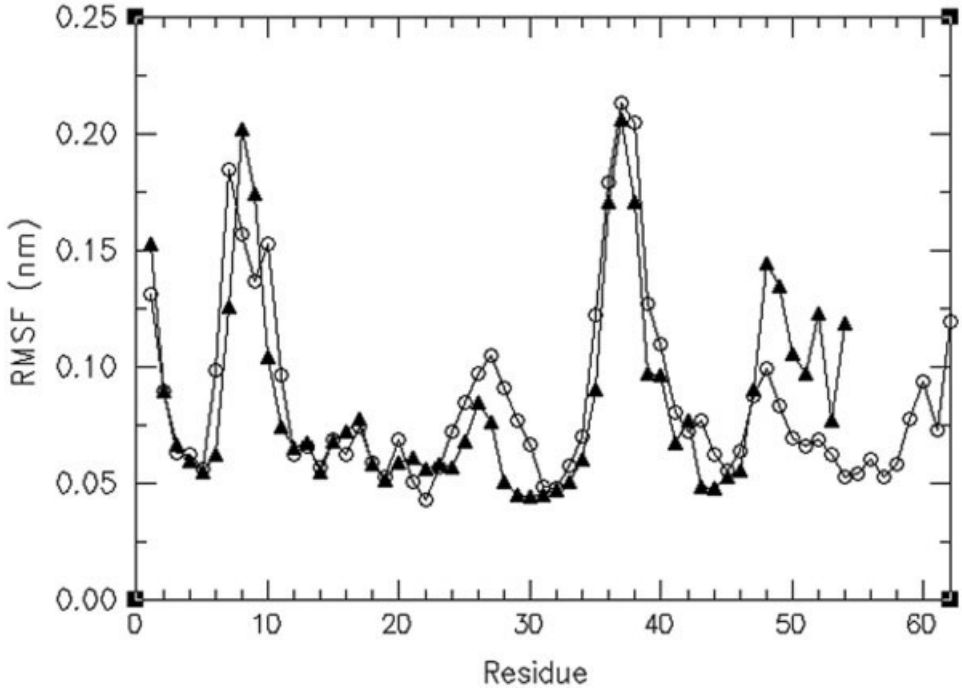

Fig. 4. Root mean square fluctuations of each amino acid of Sso7d (circles) and L54Δ (triangles) calculated from the 300-K simulation.

(see Table I). The RMSD remains stable during the trajectories, with mean values of 4.88 and 6.15 Å in the last 4 ns of the two trajectories; the C^{α} radius of gyration does not change, remaining close to the value observed at 300 K. In addition, there is no significant loss in the secondary structure content of the protein [Fig. 5(a)]. The α-helix remains fully folded, while a slight decrease in the β-sheet content is observed, associated with an increased flexibility of the β1–β2 and β5 strands (Table I). This leads to ≈15% decrease in the average number of main chain–main chain hydrogen bonds. It should also be noted that, as judged by the RMSIP calculated between the first 10 eigenvectors of the 300- and 500-K simulations, Sso7d samples similar conformational subspaces at the two temperatures (Table II).

These results agree with the strong resistance of Sso7d to high temperature experimentally detected by differential scanning calorimetry and circular dichroism.[3,14,50] Interestingly, similarly to Sso7d, the homologous Sac7d from *Sulfolobus acidocaldarius* does not unfold in a 1-ns MD simulation at 550 K performed by de Bakker and coworkers.[21] These authors attributed the resistance to high temperatures of Sac7d to the extensive ionic network characterizing the protein surface.

L54Δ partially unfolds in MD simulations at 500 K

In contrast to the parent protein, L54Δ during the two MD simulations at 500 K shows a "partial unfolding": a loss of more than 50% in the β-sheet content occurs during the 10-ns trajectories. This finding is in line with the experimental data that the denaturation temperature of L54Δ is markedly lower than that of Sso7d.[14] The β-sheet content of L54Δ is less than 20% in the last 4 ns of the trajectory, becoming 10–15% in the last nanosecond. Such a significant decrease in the secondary structure content corresponds to a complete unfolding of the strands β1, β2, and β5 [see Fig. 5(b)]. This is accompanied by a 35% decrease in the total number of main chain–main chain hydrogen bonds (40% in the last nanosecond), although the C^{α} radius of gyration of the protein is little affected (see Table I). In fact, visual inspection of structures extracted from the MD trajectories at 500 K shows that the "partial unfolded" state of L54Δ is compact and characterized by both native and nonnative contacts. This indicates that the "partial unfolded" conformations of L54Δ are as compact as the folded structure.

These results are confirmed by additional simulations performed at 500 K with a reduced water density of 0.854 g cm^{-3} (see Table III). In fact, in all MD trajectories, the β-sheet content is less than 20%, the C^{α} radius of gyration remains close to 10 Å and the total number of main chain–main chain hydrogen bonds decreases by more than 30%. It should be noted that these results are obtained with shorter MD trajectories (3–4 ns), thus suggesting, in agreement with literature data,[24,27,28] that the combination of high temperature and reduced water density leads to a more rapid unfolding.

Since a 15% reduction in the water density does not affect the average value of the radius of gyration, its constancy should not be due to the excess pressure exerted by water molecules at high temperature. The compactness of "partial unfolded" states of L54Δ is in line with the physical scenario of compact unfolded states proposed by Shimizu and Chan.[51] In addition, Knapp and co-workers

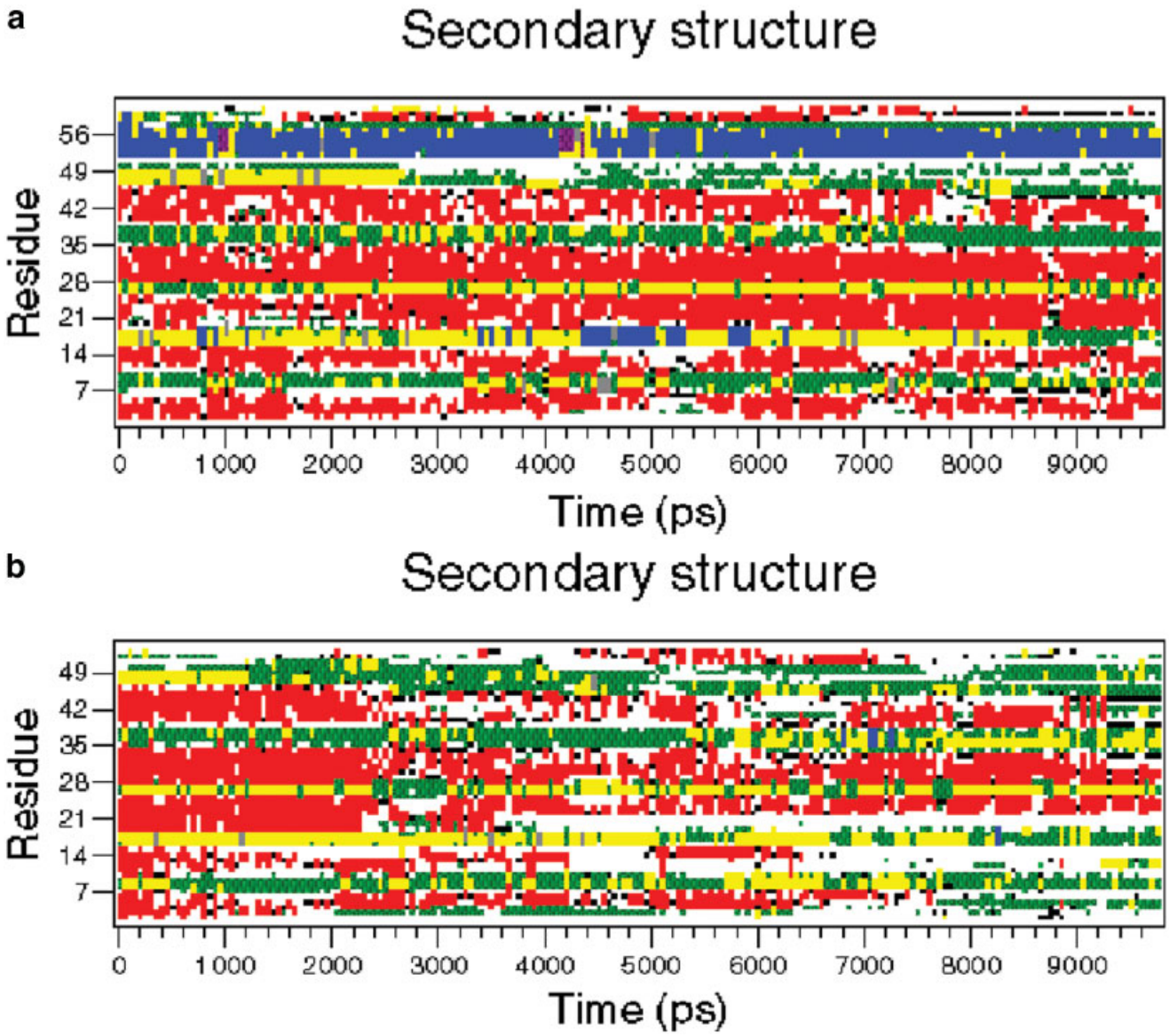

Fig. 5. Time course of the secondary structure of each residue of Sso7d (**A**) and L54Δ (**B**) in one of the two 10-ns MD simulations at 500 K. The α-helices are shown in blue, the 3_{10} helices in pink, the β-strands in red, the β-bridges in black, the bends in green, and the turns in yellow boxes.

TABLE III. Geometric Parameters Obtained From Four Different MD Simulations of L54Δ Performed at 500 K and Reduced Water Density[†]

	1	2	3	4
Simulation time (ns)	4	4	3	3
C^{α} root mean square deviation (Å)	5.65 ± 0.28	5.69 ± 0.24	5.00 ± 0.34	4.92 ± 0.27
C^{α} radius of gyration (Å)	9.35 ± 0.10	9.96 ± 0.13	10.27 ± 0.09	9.91 ± 0.14
Main chain–main chain H-bonds	15 ± 2	15 ± 3	19 ± 3	17 ± 3
β-sheet content (%)	15 ± 3	20 ± 7	17 ± 10	19 ± 7

[†]See text for more details.

did not find an increase of the radius of gyration in 12–15 ns MD simulations at 400 K of the cold shock protein from *Bacillus caldolyticus*,[52] a protein structurally similar to Sso7d and hence to L54Δ. Similarly, Roccatano and coworkers[53] found that the radius of gyration did not increase in 2.5-ns MD simulation at 550 K of horse heart cytochrome C, and Gilquin et al.[54] found, in agreement with experimental data, that the unfolded conformations of lysozyme correspond to an average radius of gyration close to that of the native state. In our opinion, these findings suggest

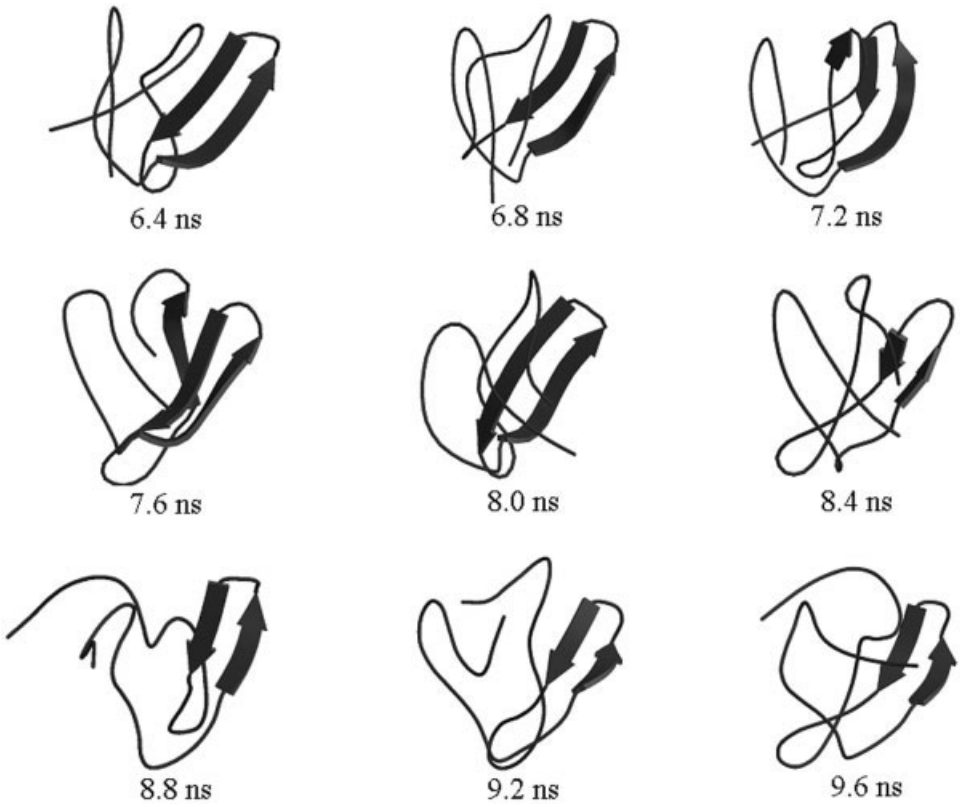

Fig. 6. Ribbon diagrams of some snapshots taken from one of the two 10-ns MD trajectories of L54Δ at 500 K.

that, in the case of compact unfolded states, both the radius of gyration and the total number of contacts are not sensitive coordinates to characterize the unfolding process. In the present case, the secondary structure content and the number of main chain–main chain hydrogen bonds seem more suitable to characterize the partial unfolded states of L54Δ.

Finally, in contrast to Sso7d, there is a clear indication that L54Δ has a different dynamic behavior at the two simulated temperatures, as emphasized by the value of RMSIP (0.53) calculated by using the first 10 eigenvectors of the 300- and 500-K simulations (Table II).

Order of events in the early phases of the thermal unfolding of L54Δ

To elucidate the sequence of molecular events occurring during the partial unfolding of L54Δ at 500 K, we have analyzed both the two 10-ns MD simulations produced with a water density of 0.997 g cm^{-3}, and four additional MD simulations produced with a water density of 0.854 g cm^{-3}. Even though the MD trajectories show some differences between each other, a defined order of events in the unfolding pathway emerged. In particular, the N-terminal and the C-terminal strands, β1–β2 and β5, respectively, are the first structural elements to undergo unfolding, whereas the central strands β3 and β4 retain a folded conformation (see, for example, Fig. 6). This suggests that the breaking of the interactions between the chain termini is the first event in the unfolding pathway. An important role in this process should be played by the salt bridge formed by Ala1 and Glu53. During the MD trajectories, the distance between the two side chains increases from 3.1 Å to an average value of about 7.5 Å, as shown in Figure 7. Interestingly, the breaking of interactions among residues located at the chain termini was recognized as the key event of unfolding by Tsai et al.[55] from MD simulations of src SH3, and by Gsponer and Caflish[56] from MD simulations of four SH3 domains, in agreement with the experimental studies.[57,58] These results lend support to the hypothesis that globular proteins with similar folding topologies should have similar unfolding pathways.[56,58]

DISCUSSION

MD simulations at 300 and 500 K have been performed to study the structural and dynamic effects of the C-terminal α-helix truncation on Sso7d. The results of the simulations at 300 K showed that, as suggested by experimental evidence,[14] the truncated variant assumes a folded structure, similar to that of the corresponding part of the parent protein, in particular in the region involved in the DNA binding. Thus, the C-terminal helix does not appear essential for the correct folding of the β-barrel of Sso7d and so for the DNA recognition. Furthermore, the deletion of the last 8 residues does not produce dramatic changes in global motions of L54Δ compared to Sso7d, but generates a slightly increased local flexibility in the region close to the truncation site, residues 48–54. These results indicate that L54Δ and Sso7d have similar structures and dynam-

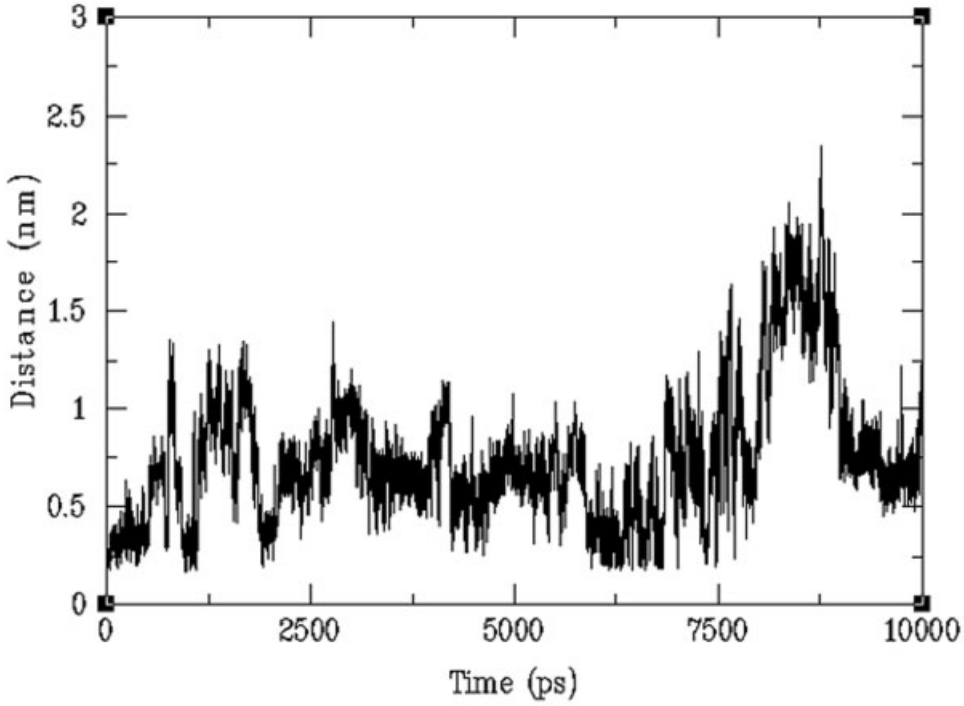

Fig. 7. Plot of time course of the distance between the side chains of Ala1 and Glu53 during one of the 10-ns MD trajectories of L54Δ.

ics at 300 K, supporting the notion that proteins with analogous fold exhibit similar overall motions.[43] On this ground, the marked difference in thermal stability between the two proteins should be searched in the different behavior at high temperatures, rather than in a difference in the intrinsic flexibility of the folded state at room temperature.

MD simulations at 500 K suggest that the fluctuations of the C-terminal residues 48–54 in L54Δ should play a fundamental role in the unfolding. The amplification of such fluctuations at high temperature produces a rapid breaking of the interactions between the N-terminal and C-terminal strands. In this respect, it is important to note that in the structure of Sso7d these two regions of the chain are linked by a strong salt bridge between the carboxyl oxygen of Glu53 and the amino terminal group of Ala1 (3.1 Å; see the dotted line in Fig. 1).[8] This interaction seems fundamental for the conformational stability of Sso7d: a variant truncated at Glu53 could not be isolated under the same conditions that allowed the isolation and the purification of L54Δ (Prof. Paolo Tortora, personal communication). In L54Δ, this salt bridge should be assured by Leu54 whose side chain is well buried and anchored to the hydrophobic core of the protein (see Fig.1), although it should undergo large fluctuations (see Fig. 7) being close to the truncation site. Therefore, the rupture of the Ala1-Glu53 salt bridge and of the interactions involving the Leu54 side chain should be the first event in the thermal unfolding of L54Δ, leading to the unzipping of the N-terminal and C-terminal strands, β1, β2, and β5. These results are in line with the order of unfolding events detected for several SH3 domains,[55–58] and support the conclusion that proteins with the same native topology exhibit similar early stages of unfolding pathways.[56,58]

The MD simulations of L54Δ at 500 K also show that the strands β3 and β4 are resistant in-silico to thermal unfolding, pointing out that these strands could be the "nucleus" for the folding of the protein. This agrees with the results from the experimental Φ-value analysis by Guerois and Serrano,[32] indicating the strands β3 and β4 as the folding nucleus of Sso7d. The burial of several hydrophobic residues occurring in this region of the chain (Val22, Val25, Met28, Ile29, and Phe31) should be the main driving force for the folding and stability of the protein. In line with this hypothesis, the single point mutation F31A in Sso7d causes a dramatic destabilization of the protein with respect to both temperature and pressure.[50,59]

The MD trajectories of Sso7d at 500 K indicate that the amphipathic C-terminal α-helix is always fully folded. This finding implies that the presence of the C-terminal helix limits the flexibility of the region 48–54, through an optimization of hydrophobic interactions in the core and of electrostatic interactions on the surface, and so prevents the breaking of the inter-termini interactions, which appear involved in the early phases of the unfolding. Therefore, the present study points out that the C-terminal helix is not essential for the correct folding of the β-barrel region of Sso7d and for the DNA-binding activity, but plays a fundamental role for the resistance to high temperature of the protein. The tight anchoring of the chain termini to each other and/or to the protein core appears to be an important stabilizing mechanism of Sso7d and, in all probability, of several other small globular proteins.

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