Stability and Unfolding Mechanism of the N-terminal β-Hairpin from [2Fe-2S] Ferredoxin I by Molecular Dynamics Simulations

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The stability and unfolding mechanism of the N-terminal β-hairpin of the [2Fe-2S] ferredoxin I from the blue-green alga Aphanothece sacrum in pure methanol, 40% (v/v) methanol-water, and pure water systems were investigated by 10 ns molecular dynamics simulations under periodic boundary conditions. The β-hairpin was mostly in its native-like state in pure methanol, whereas it unfolds dramatically following the ‘zip-up’ mechanism when it was placed in pure water. Both interstrand and inside-turn hydrogen bonds account for the stability of the β-hairpin in its native-like conformation, whereas hydrophobic interactions among nonpolar side chains are responsible for maintaining its stable loop-like intermediate structures in 40% (v/v) methanol-water. Reducing solvent polarity seems to increase the stability of the β-hairpin in its native-like structure. Methanol is likely to mimic the partially hydrophobic environment around the N-terminal β-hairpin by the subsequent α-helix.

Keywords: Blue-green alga Aphanothece sacrum; Methanol; Periodic boundary conditions; ‘Zip-up’ mechanism; Hydrogen bonds; Hydrophobic interactions; Polarity.

INTRODUCTION

As the knowledge of various DNA and protein sequences from different organisms rapidly increases, proteomics has become extremely important in the post-genomic era. The tasks of proteomics can be categorized into three main activities: 1) identifying all the proteins made in a given cell, tissue or organism; 2) determining how those proteins join forces to form complicated networks; and 3) outlining the precise three-dimensional structures of the target proteins for further rational drug design. Both x-ray crystallography and NMR spectroscopy have been widely used for the structural determination of proteins. In addition, much attention has been paid to investigate the folding/unfolding mechanisms of proteins under different conditions. Ever since the early experiments, protein folding has been recognized to be a spontaneous event and all the information required for correct folding is believed to be contained in the amino acid sequence. Different folding models (e.g., nucleation, early secondary structure formation, or hydrophobic cluster formation) have been proposed so far to explain the complicated folding mechanisms. However, according to the Levinthal paradox, protein folding cannot occur by a random search of all possible conformations.

Since numerous advances by successful design of certain model peptides to understand protein structure and folding mechanisms have been achieved in the past few years, the study of small peptides and protein fragments leads to a better understanding of protein folding in the early stage, where both β-turns and β-hairpins have been considered to act as the folding initiation sites. Examples were given by the investigation of the early events of protein folding with isolated peptide fragments under equilibrium conditions. Previous studies with fragments from protein-GB1 domain (GB1) and ubiquitin presented the autonomous folding of short peptide molecules into β-hairpins with various stability in aqueous and mixed aqueous-organic solvent systems. Moreover, it has been indicated by computer molecular simulations that the time for β-turn and β-hairpin peptides to fold into their native state is within ns and μs, respectively. These promising results have made it feasible to conduct the protein folding/unfolding processes ‘in Silico’.

The stability of the β-hairpin in aqueous solution has been attributed to many factors. For example, alcohols have been shown to occasionally stabilize the structure of β-hairpin peptides due to the relatively local hydrogen bond interactions. Local hydrogen bonds seem to play an important role for secondary structure stabilization in solvents of relatively low polarity, whereas the hydrophobic interactions show less influence on stabilizing a protein. The effect of al-

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cohols can also be explained to some extent by decreased solvent polarity.\textsuperscript{37-40} In addition to the importance of solvent properties, intrinsic residue preferences and interstrand interactions have also been showed not only to promote $\beta$-hairpin formation from various linear peptides,\textsuperscript{20,22,41,42} but also to contribute to the $\beta$-hairpin stability significantly.\textsuperscript{43-45} Furthermore, the $\beta$-turn residues can also be crucial to the stabilization of $\beta$-hairpin structure.\textsuperscript{46-48} Both type I’ and type II’ $\beta$-turns are the most abundant turn motifs found in $\beta$-hairpins so far,\textsuperscript{49} probably due to that these turns exhibit a favorable geometry to match the right-handed twist of the anti-parallel $\beta$-sheet.\textsuperscript{50}

Previous study has shown that the folding/unfolding of a 19-residue peptide, corresponding to the first $\beta$-hairpin of the $\alpha$-amylase inhibitor tendamistat, follows the so-called ‘zip-up’ mechanism (Fig. 1), in which the $\beta$-turn is formed first followed by hydrogen bond formation closing the hairpin, and subsequent stabilization by side-chain hydrophobic interactions.\textsuperscript{25} In addition, Maynard \textit{et al.}\textsuperscript{51} have suggested that the hydrophobic effect is the dominant stabilizing interaction for $\beta$-hairpin in water by structural and thermodynamic analyses of the model peptide. In the present study, we examined the solvent effects on the stability and the unfolding mechanism of the N-terminal $\beta$-hairpin of the [2Fe-2S] ferredoxin I from the blue-green alga \textit{Aphanotoce sa crum} by molecular dynamics simulations in different aqueous-organic solutions.

The structure of the [2Fe-2S] ferredoxin I from the unicellular blue-green alga \textit{A. sacrum} has been determined by x-ray crystallography.\textsuperscript{52} It is composed of four identical subunits; each of them consists of 96 amino acids and has a relative molecular weight of 10.48 kDa (Fig. 2). The amino acid sequence of this protein was found to differ from that of the [2Fe-2S] ferredoxin from the filamentous blue-green alga \textit{Spirulina platensis}\textsuperscript{53,54} by only 30\%,\textsuperscript{52} leading to the observa-

![Fig. 1. The possible folding/unfolding mechanisms of $\beta$-hairpin of Tendamistat proposed by Bonvin \textit{et al.}\textsuperscript{25} The ‘Zip-up’ mechanism shows that the $\beta$-turn is formed first, followed by hydrogen bond formation closing the $\beta$-hairpin. In contrast, the interstrand hydrogen bonds are formed first, followed by forming a stable $\beta$-turn structure for the ‘Zip-down’ mechanism.](image1)

![Fig. 2. The ribbon structure of one of the four subunits of [2Fe-2S] ferredoxin I.\textsuperscript{52} The N- and C-terminus are indicated as N and C, respectively. The N-terminal $\beta$-hairpin and the subsequent $\alpha$-helix are shown in dark and light blues, respectively.](image2)

![Fig. 3. The stereo view of the N-terminal $\beta$-hairpin of [2Fe-2S] ferredoxin I.](image3)
tion that both enzymes share a β-barrel structure and adopt a common α/β-roll fold, consisting of four β-strands and one α-helix. This fold is similar to the structure motif in both ubiquitin and GB1. The four β-strands in [2Fe-2S] ferredoxin I from the blue-green alga A. sacrum are formed by the residues Tyr2-Thr9, Gly12-Val20, Lys50-Val52 and Asp84-Glu88 and the α-helix is formed by the residues Tyr-23-Glu31. Both ubiquitin and [2Fe-2S] ferredoxin I adopt a hairpin-turn-helix motif where the two β-strands near the N-terminus form a stable β-hairpin structure connected to the subsequent α-helix, which forms a large proportion of the hydrophobic core of the proteins. This hairpin-turn-helix motif has become a good model system to investigate the stability of the β-hairpin structure in a hydrophobic environment.

RESULTS AND DISCUSSION

In the present study, the 20-residue peptide, ASYKVTLKTPDGDNVITVPD, corresponding to the N-terminal β-hairpin from residues 1 to 20 of the [2Fe-2S] ferredoxin I from the unicellular blue-green alga A. sacrum taken from the x-ray crystallographic structure (PDB entry F1XI) was subjected to 10 ns MD simulations in different solvent systems. To investigate the influence of different solvents on the stability of the initial structure, we submitted the target β-hairpin fragment, which was first energy minimized by steepest descent method for 5,000 iterations, into the pure methanol, 40% (v/v) methanol-water, and pure water systems, respectively. Fig. 4 shows that the starting structure of β-hairpin in pure methanol is similar to the crystallographic structure, whereas it significantly deviates from the crystallographic structure when it was placed in the pure water system. The RMSDs of the backbone Cα of the starting structure in the pure methanol, 40% (v/v) methanol-water, and pure water systems with reference to the crystallographic structure are 0.6, 2.1, and 2.0 Å, respectively (Fig. 5). These results in-
dicate that solvent does play an important role for secondary structure stabilization, which is consistent with the previous CD and NMR spectroscopy results showing that addition of methanol or TFE to an aqueous solution of this β-hairpin fragment promotes the formation of native-like monomeric β-hairpin structure. The effect of alcohols on stimulating secondary structure formation has been mainly attributed to a strengthening of intramolecular hydrogen bonding. The lower dielectric constant in a methanol system seems to result in less effective solvation of amide dipoles than pure water, leading to the stabilization of the folded state of β-hairpin structure where intramolecular amide hydrogen bonds are energetically more favorable than amide-solvent intermolecular hydrogen bonds.

As can be seen from the crystallographic structure, the N-terminal β-hairpin is subsequently connected by an α-helix. It is believed that additional interactions of the β-hairpin with the α-helix and other portions of the polypeptide chain as prerequisites for secondary structure stabilization are necessary. The observation that the β-hairpin structure resembles its crystallographic structure in the pure methanol system, whereas it shows a dramatic deviation from its native-like structure in the 40% (v/v) methanol-water and pure water systems, strongly indicates that the less polar solvent system is likely to mimic the hydrophobic environment surrounding the N-terminal β-hairpin provided by the subsequent α-helix, leading to stabilizing the β-hairpin structure. Methanol stabilizes the β-hairpin structure mainly by strengthening amide-amide hydrogen bonds relative to amide-solvent interactions.

As can be seen from Fig. 6(a), the total number of intramolecular hydrogen bonds varied between 5 and 11 during the 10 ns MD simulations in the pure methanol system. Here, we define the interstrand hydrogen bonds as the hydrogen bonds formed between the amide and carbonyl groups of residues Tyr3 and Val18, Val15 and Ile16, and Leu7 and Asn14. The inside-turn hydrogen bonds are defined as the hydrogen bonds formed between charged or polar side chains located on the same face of the β-hairpin. Fig. 6(a) shows that the number of the inside-turn hydrogen bonds remained at the level of 2 during most of the MD simulation course, while the number of the interstrand hydrogen bonds varied between 3 and 6. It indicates that hydrogen bonds close to the β-turn region are more stable than those located far away from the β-turn. Pro10 located in the β-turn is likely to reduce the degree of freedom of the backbone near the β-turn region due to the conformational restriction effect by its cyclically bonded structure, leading to stabili-

Fig. 6. The number of hydrogen bonds formed in the 10 ns MD simulations in (a) the pure methanol system; (b) the 40% (v/v) methanol-water system; and (c) the pure water system. : the total number of hydrogen bonds; : the number of the interstrand hydrogen bonds; ×: the number of the inside-turn hydrogen bonds; : the number of interside-chain hydrogen bonds in the two β-strands; : the number of interside-chain hydrogen bonds in the β-turn region.
lizing the hydrogen bonds formed near this region. The interside-chain hydrogen bonds do not contribute to stabilize the β-hairpin structure since only 1 or 2 were found during the entire MD simulation course.

Although the possibility to solve the protein folding problem by direct in silico simulations on the atomic scale has not yet become a reality due to the relatively small time-span, the increased computer power has started to make some of the relevant problems in protein folding amenable to simulation methods such as molecular dynamics (MD) and Monte Carlo (MC) calculations. While the latter method has been primarily applied in the context of exploring simplified lattice or grid models of protein structure, the former one has been widely employed to gain insight into the protein folding/unfolding processes. Protein folding usually occurs on the second time-scale, which has made computer simulations difficult to reach the realistic situations. To overcome this problem, the so-called unfolding simulations have started to attract intensive investigation since the early 90s. The common strategies to induce the unfolding of a protein in its native-like structure are the temperature jump technique, pressure-induced denaturation, and changing the solvent. However, thermal denaturation is not necessarily similar to protein folding at physiological or room temperatures with respect to possible folding/unfolding pathways, intermediates sampled, and transition states. Therefore, protein folding/unfolding induced by different solvents has provided an alternative approach to investigate the possible protein folding/unfolding mechanisms either by in vitro experiments or by in silico simulations. Daura et al. had shown that a short β-heptapeptide can fold into its native 3-helical structure spontaneously in methanol on a 10 ns time-scale simulation. This promising result encourages us to investigate the possible unfolding mechanism of the target β-hairpin induced by solvent effects with MD simulations.

Fig. 7 shows the snapshots of the β-hairpin structure superimposed on the crystallographic structure from a 10 ns MD simulation in the pure methanol system. As can be seen, the target β-hairpin maintains a stable conformation that is closely similar to its native-like structure during the entire course of the 10 ns MD simulation. The two β-strands of the β-hairpin remained connected by interstrand hydrogen bonds,
whereas the \( \beta \)-turn region deviated from its relevant position in the crystallographic structure after 2 ns of simulation. Although Gly12 located next to the \( \beta \)-turn region is likely to increase the backbone flexibility in the \( \beta \)-turn region, it still remains in the well defined type I' \( \beta \)-turn motif throughout the whole 10 ns simulation by forming two stable hydrogen bonds between the amide and carbonyl groups of residues Thr9 and Gly12. It indicates that interstrand hydrogen bonding along with the stability of the \( \beta \)-turn play an important role in keeping \( \beta \)-hairpin in its stable native-like conformation. Fig. 7 also shows that some of the side chains are free to fluctuate except for Tyr3, Val5, Ile16, and Val18. The hydrophobic interactions between the nonpolar side chains of Tyr3 and Val18, and Val5 and Ile16 restrict the movement of these side chains, leading to contribute to the stabilization of the \( \beta \)-hairpin structure in pure methanol. The present simulation result is consistent with the previous NOEs observations, in which some residue side chains located on the same face of the \( \beta \)-hairpin were shown to potentially stabilize the structure through the hydrophobic interaction or van der Waals packing.\(^{25}\) Fig. 5(a) shows that the RMSDs of the backbone \( C_\beta \) for the \( \beta \)-hairpin in pure methanol fluctuate between 0.6 and 1.3 Å during the 10 ns MD simulation. This result also strongly indicates that the structure of the \( \beta \)-hairpin is very stable in the pure methanol system.

Compared with the stabilization effect in pure methanol as shown in Fig. 7, the \( \beta \)-hairpin started to unfold in the 40% (v/v) methanol-water system as observed in Fig. 8. The snapshots at 4 and 6 ns present the two intermediate structures, which show more deviations than the intermediate structures sampled at other time points. Similar results can also be seen from Fig. 5(b), in which the RMSD of the backbone \( C_\beta \) versus MD simulation time was presented. The RMSDs varied between 2 and 4 Å during the simulation time course and reached the maximal value between 4 and 6 ns. 

Previously, Bonvin \textit{et al.}\(^{25}\) conducted MD simulations of the reduced and oxidized native 19-residue peptide corresponding to the first \( \beta \)-hairpin of tensamistat and showed that only a limited portion of the accessible intermediate conformations in the transition state need to be sampled. In the present study, the \( \beta \)-hairpin in the 40% (v/v) methanol-water system reveals the possible intermediate conformations in the transition state during the folding/unfolding processes. As can be seen in Fig. 10, the stable intermediate conformation sampled by the \( \beta \)-hairpin is the loop-like structure maintained through the hydrophobic interactions among the nonpolar side chains of residues Val5, Leu7, Ile16, and Val18. The introduction of water in the 40% (v/v) methanol-water system results in a strong solvation effect, leading to destruction of the amide-amide hydrogen bonds and consequently destabilizing the \( \beta \)-hairpin structure. Meanwhile, the nonpolar groups of the methanol molecules interact with the nonpolar side chains of residues Val5, Leu7, Ile16, and Val18 and subsequently orient them into the interior region of these two strands (Fig. 10). The strong hydrophobic interactions among these side chains allow these two \( \beta \)-strands to stay close to each other even after they have lost most of the interstrand hydrogen bonds. It has been previously shown that hydrophobic interactions among side chains play an important role in stabilizing a model peptide conformation in water by thermodynamic and structural analyses.\(^{51}\) In the present study, we found that the hydrophobic effect to stabilize the secondary structure in solution is more significant in the 40% (v/v) methanol-water system than in the pure methanol system. As can be seen in Fig. 6(b), the total number of hydrogen bonds varied between 0 and 5 in the 40% (v/v) methanol-water system during the 10 ns MD simulation. The total hydrogen number is usually smaller than 3 between 4 and 6 ns. The numbers of both interstrand and inside-turn hydrogen bonds remained 1 most of the time in the 40% (v/v) methanol-water system.
system, which means that about 80 and 50% of the original hydrogen bonds have disappeared in the strand and turn regions, respectively. It again indicates that the β-turn region is more stable than the β-strand region while β-hairpin starts unfolding induced by the solvent with higher polarity. Comparing the pure methanol system to the 40% (v/v) methanol-water system, we can conclude that both the interstrand and inside-turn hydrogen bonds play a major role in stabilizing the β-hairpin structure when it is in its native state, whereas hydrophobic interactions among the nonpolar side are responsible for the intermediate structure stabilization when β-hairpin is in its partially unfolded state.

Fig. 9 shows the snapshots of the target peptide in the pure water system during the 10 ns MD simulation. We found that the structure dramatically unfolded in the so-called ‘zip-up’ manner (Fig. 1),25 in which the two β-strands opened first, followed by the destruction of the β-turn region. The strong solvation free energy required to replace the amide-amide hydrogen bonds by the energetically more unfavorable amide-water interactions is compensated by the increased entropy during the spontaneous unfolding process. Fig. 6(c) shows the numbers of hydrogen bonds versus simulation time for β-hairpin in the pure water system. The total number of hydrogen bonds decreased dramatically. Some residual inter-side chain hydrogen bonds still can be found during the simulation, whereas most of the interstrand and inside-turn hydrogen bonds disappeared. The RMSDs of the backbone Cα for β-hairpin in the pure water system increased dramatically from 2 Å at the starting point to about 14 Å at 10 ns (Fig. 5(c)). This again indicates that the β-hairpin unfolds rapidly in the pure water system.

Comparing Figs. 7-9, we found that the β-hairpin is maintained in its native state in the pure methanol system, whereas it unfolds spontaneously following the ‘zip-up’ mechanism25 (Fig. 1) in the pure water system. It is noteworthy that the β-hairpin partially unfolds and stays in the stable intermediate state in which the nonpolar side chains of residues Val5, Leu7, Ile16, and Val18 protrude into the interior of the two β-strands and the resulting hydrophobic interactions turn the β-hairpin into the like-loop structure. The present result that methanol stabilizes the β-hairpin in its native-state structure is consistent with the previous findings that the native-like β-hairpin structure can be induced by reducing the solvent polarity by addition of methanol or TFE observed by CD and NMR spectroscopy.18 The pure methanol system is likely to mimic the partially hydrophobic environment around the β-hairpin provided by the subsequent α-helix in the [2Fe-2S] Ferredoxin I subunit. It emphasizes the importance of additional interactions with other portions of the polypeptide chain for stabilizing the secondary structure and promoting the folding of the correct tertiary structure of the protein.72-74

EXPERIMENTAL

The initial structure of the 20-residue peptide sequence, ASYKVTKTPDGDNVITVPD (Fig. 3), corresponding to the N-terminal β-hairpin from residue 1 to 20 of the [2Fe-2S] ferredoxin I from the unicellular blue-green alga A. sacrum...
was obtained from the x-ray crystallographic structure (PDB entry F1XI). There are 8 interstrand hydrogen bonds in the original crystallographic β-hairpin structure (Table 1). The MD simulations were conducted in the SGI O200 workstation (Silicon Graphics, Inc., Mountain View, CA, USA)–Insight II (Accelrys, San Diego, CA, USA) system with the force field Discover CVFF (consistent valence force field). The initial structure was first energy minimized by the slow but robust steepest descent method with 5,000 iterations to be used as the starting structure for further structural comparison. Three 35*35*35 Å$^3$ cubic lattices, with the first one containing 1315 water molecules, the second one containing 528 methanol molecules, and the third one containing 847 water and 211 methanol molecules, were constructed for the pure water, pure methanol, and 40% (v/v) methanol-water systems, respectively. The starting structure was injected into the centers of these lattices for 10 ns MD simulations using the Insight II program. The time-step used in the MD simulations was 2 fs. The temperatures and pressures of these systems were maintained constant by weakly coupling the target peptide, solute, and solvent, separately, to an external temperature bath at 300 K and to an external pressure bath at 1 atm, respectively. All non-bonded interactions within the 8-Å cut-off radius were calculated for each MD time step, while the long-range contributions, between the radii of 8 and 211 Å, were updated every five steps. Position restraints were applied on the solute heavy atoms during the equilibrium stage of the simulation to allow the solvent (e.g., water and methanol) to equilibrate without disturbing the target peptide structure. The trajectories and coordinates of the peptide molecule were saved every 100 ps for further analysis. All simulations were performed on an SGI O200 system with 64-bit HIPS RISC R12000 270 MHz CPU and a PMC-Sierra RM7000A 350 MHz processor. About 1s of CPU time was required for one 2 fs simulation, leading to a total running time of 120 to 130 CPU days for each of the three systems.

Although some complicated algorithms have been proposed to measure the structural similarity between proteins, the root-mean-square deviation (RMSD) remains the most commonly used. For each MD simulation, the RMSDs of the trajectories recorded every 100 ps interval were calculated for the backbone C$_\alpha$ atom of the β-hairpin during the course of 10 ns MD simulations at 300 K with reference to the starting structure, which has been previously energy minimized. As a result, RMSD is a useful measure of structural similarity for closely related proteins or for the structural change during protein folding/unfolding processes. The RMSDs were calculated after optimal superimposition of the coordinates to remove translational and rotational motion. The starting structure of the MD simulations, which was generated from about 10 ps equilibration, generally had a very similar folding structure to that of the initial crystallographic structure with RMSD < 0.2 Å (data not shown). The hydrogen bonding was predicted based on the Kabsch-Sander algorithm, in which pattern recognition of hydrogen bonds was correlated to the geometrical features. The default hydrogen bonding energy criterion of -0.5 kcal/mol was used.

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Abbreviations

H-bond, hydrogen bond; MD, molecular dynamics; RMSD, root-mean-square deviation.

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REFERENCES

17. Searle, M. S.; Williams, D. H.; Packman, L. C. Nature...


