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Dynamical Properties of Fasciculin-2

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ABSTRACT **Fasciculin-2 (FAS2) is a potent protein inhibitor of the hydrolytic enzyme acetylcholinesterase. A 2-ns isobaric-isothermal ensemble molecular dynamics simulation of this toxin was performed to examine the dynamic structural properties which may play a role in this inhibition. Conformational fluctuations of the FAS2 protein were examined by a variety of techniques to identify flexible residues and determine their characteristic motion. The tips of the toxin "finger" loops and the turn connecting loops I and II were found to fluctuate, while the rest of the protein remained fairly rigid throughout the simulation. Finally, the structural fluctuations were compared to NMR data of fluctuations on a similar timescale in a related three-finger toxin. The molecular dynamics results were in good qualitative agreement with the experimental measurements. Proteins 1999;36:447–453.**
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Key words: fasciculin; acetylcholinesterase; molecular dynamics

INTRODUCTION

Fasciculin-2 (FAS2), a member of the three-finger toxin family, is known to be a nanomolar inhibitor of acetylcholinesterase (AChE),[1–3] an enzyme that hydrolyzes neurotransmitters at cholinergic synapses.[4,5] Since AChE is a target for the synthesis of pesticides, pharmaceuticals, and nerve agents,[5,6] FAS2 has been the center of many experimental studies to determine the nature of its interaction with AChE. Simulations of FAS2 can help provide insight into this interaction and experimental studies by uncovering the microscopic motion of FAS2 on the nanosecond timescale.

The crystal structures of the free FAS2 protein,[7] as well as the FAS2-Torpedo californica (electric ray) AChE[8] and the FAS2-mouse AChE[9] complexes, have been solved and provide detailed insight into the interaction between the two proteins. Fasciculin-2 (see Fig. 1) contains 61 amino acids structured into a core region with three β-sheet loops, or the "fingers" of the toxin formed by residues 3–15, 22–39, and 40–53, respectively.[7] This protein also has four disulfide bonds, which contribute to the core and loop stabilities.[7] As the FAS2–AChE crystal structure and other experimental evidence indicate, residues 30–33 of loop II play the key role in binding to the peripheral anionic site of AChE,[2,9–11] located at the entrance to the gorge that leads to the active site, during FAS2 inhibition. A second interaction between FAS2 and AChE is formed via the contact of residues 7–12 at the tip of loop I with a depression in the AChE surface 2.5 nm away from the active-site gorge entrance.[9] Finally, residues Tyr 4 and Tyr 61 interact with AChE residues Tyr 77 and Pro 78 via van der Waals contacts.[9] Overall, 27% of the FAS2 molecular surface contributes to the 11 nm^2 area of the FAS2-AChE interface.[9]

Based on comparisons between free[12] and complexed fasciculin proteins,[9] several regions of the FAS2 molecule are thought to undergo conformational changes upon binding to AChE. Although the related three-finger toxin, fasciculin-1 (FAS1), differs from FAS2 by only one residue,[12] the crystal structures of FAS2 have significant structural differences from the uncomplexed FAS1 X-ray structure. For example, the conformation of loop I in the crystal structure of uncomplexed FAS2 is shifted by several angstroms from the FAS1 structure by the presence of a detergent molecule.[7] The resulting conformation of loop I in the uncomplexed FAS2 structure closely resembles its conformation in the FAS2-AChE complex, which also differs significantly from the FAS1 structure.[12] This evidence suggests significant flexibility for loop I. Finally, the crystal structure of the FAS2-AChE complex protein also shows the tips of the loops I and II shifted by 0.2–0.25 nm with respect to a free FAS1 structure.[9] Molecular dynamics (MD) simulations provide a method to probe for the dynamical properties resulting from these flexible regions of the protein. This information could be used to create a basis for a detailed examination of the encounter and interaction of FAS2 with AChE during inhibition.

METHODS

The 2.0-Å crystal structure of fasciculin-2, isolated from venom of the green mamba snake, was obtained from the Protein Data Bank (1fsc).[7] This structure resolved all 61 residues of monomeric fasciculin-2 cocrystallized with β-octyl glucoside as well as 56 water molecules. As mentioned previously, the presence of the detergent molecule perturbed the structure of loop II in fasciculin-2 from its

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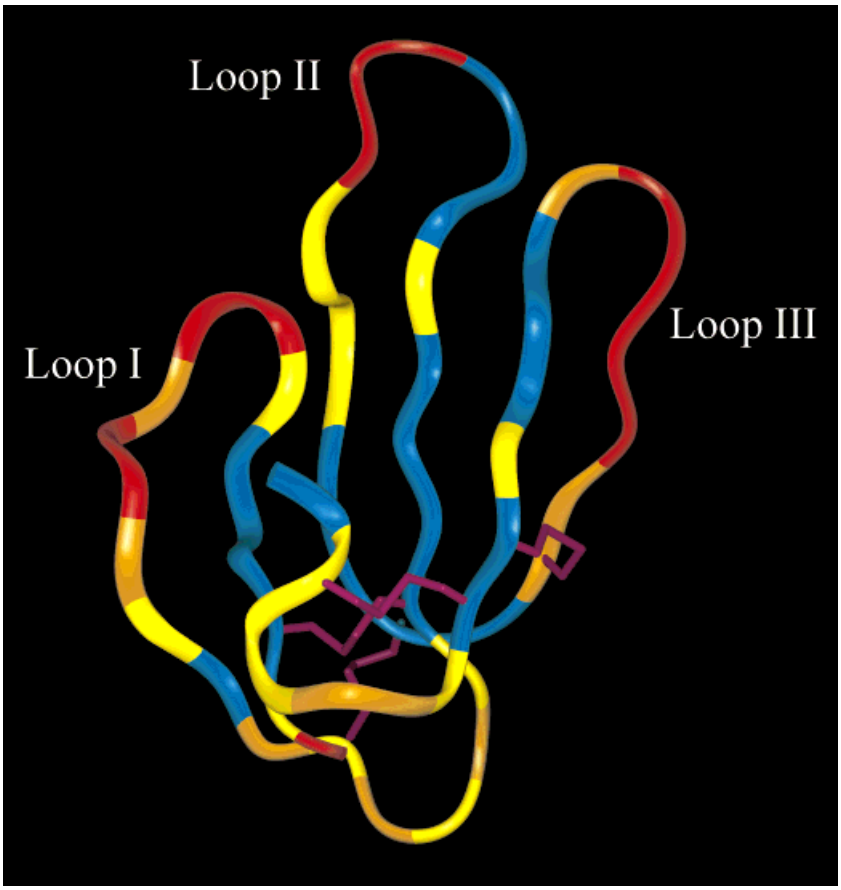

Fig. 1. Backbone worm of FAS2 protein with amide fluctuations shown by color, where red residues are the most flexible and blue residues are the least. Red residues correspond to nitrogen atoms with relative mean square fluctuations greater than 0.35, orange to fluctuations in the range 0.2–0.35, yellow to the range 0.1–0.2, and blue to fluctuations less than 0.1. The disulfide bonds (purple) that contribute to the core stability are also shown.

conformation in the homologous FAS1[7,12] to a position similar to that in the FAS2-AChE complex.[9] Although the atoms of this detergent molecule present in the crystal structure were removed prior to the simulation setup, all other crystallographic atoms, including the water molecules, were preserved throughout the setup.

The University of Houston Brownian Dynamics (UHBD)[13,14] software package was used to determine the protonation states of titratable residues in the protein. Results from both the cluster[15] and the Monte Carlo methods[16] for titration state analysis gave very similar results. Specifically, all Arg, Lys, Glu, and Asp residues were assigned charged states, while all His and Tyr residues were protonated in a neutral fashion. In this titration state, the overall charge of fasciculin-2 was +4 e.

The molecular analysis program WHAT IF[17] was used to place polar hydrogens on both the protein atoms and crystallographic water oxygens. In general, the protonation scheme of WHAT IF gave results consistent with the UHBD titration analysis. However, WHAT IF doubly protonated His 29 in contradiction to the UHBD results. Inspection of the protein environment surrounding this residue indicated the most favorable hydrogen bonding pattern via protonation of the ϵ nitrogen of the His 29 imidazole ring. Accordingly, the δ proton assigned by WHAT IF was removed to give a neutral His 29 consistent with the UHBD titration state. After the polar hydrogens were positioned by WHAT IF, the NWChem 3.2 software package[18] was used to add aliphatic hydrogens and to refine the hydrogen atom positions. This energy refinement was performed using charges assigned by the AMBER 95[19] force field with the SPC/E model for the crystallographic water molecules.[20]

In order for electrostatic interactions to be calculated with the particle-mesh Ewald (PME) technique, the system must be electrically neutral. For this reason, 4 chlorine ions were placed 0.5–1.0 nm from the protein surface in arbitrary positions. Since the dimensions of the protein are roughly 3.5 nm $\times$ 2.5 nm $\times$ 3.0 nm, a cubic periodic simulation box of 5.3 nm sides was chosen. At this box size, all copies of the protein related by periodicity were separated by at least 1.6 nm. In addition to the crystallographic water, the protein and ions were solvated with 4,452 SPC/E waters using the NWChem 3.2 program, giving a total of 4,508 SPC/E water molecules. Including the solvent, 61 protein residues, and 4 ions, the overall number of simulated atoms was 14,412.

While the previous steps in the simulation setup were carried out on either Silicon Graphics or Linux Intel platforms, subsequent setup steps and the actual simulation were performed with NWChem 3.2 and 3.2.1 on 16 nodes of a 256-node Cray T3E. All MD simulations described in this paper were performed under periodic boundary conditions with PME treatment of electrostatic interactions using a 1.0 nm cutoff radius. Bonds between

heavy atoms and hydrogens were constrained with the SHAKE algorithm.[21]

The first step in equilibration of the system was energy relaxation of the solvent molecule positions. The atoms of the protein and ions were then fixed and the solvent was subjected to 20 ps of constant volume and temperature (NVT) MD[22] at 298.15 K using 2 fs steps. Although some energy components, such as solvent-solvent Lennard-Jones interactions, had not completely converged after this period of dynamics, the overall solvent energy was sufficiently stable to continue with equilibration of the remaining system. Next, the energies of the protein atoms and ions were relaxed for 100 steps by a steepest descent algorithm while the solvent positions were held constant. This was followed by a series of short NVT MD runs at successively higher temperatures designed to slowly equilibrate the entire system to the simulation temperature. Unless otherwise noted, these simulations were carried out with 2 fs time steps and velocity reassignment to maintain a reasonably uniform temperature throughout the system. The dynamics schedule was 1.25 ps at 50 K with 0.5 fs steps, 2.5 ps at 50 K, 3.5 ps at 100 K, 4.5 ps at 150 K, 5.5 ps at 200 K, 6.5 ps at 250 K, and finally, 7.10 ps at 298.15 K with no velocity reassignment. As expected, the initial dynamics run at 50 K showed little convergence in the energetic properties. However, the subsequent MD runs showed good equilibration in this regard. Finally, the system was subjected to a final 25 ps of dynamics without velocity reassignment at 298.15 K in a constant pressure and temperature (NpT) ensemble to complete the initial equilibration.[22] The production runs began after this period using the same dynamics conditions. Specifically, these runs were performed at 298.15 K with 2 fs steps in an NpT ensemble with no velocity reassignment. The production runs were performed for a total of 2,180.2 ps, or roughly 300 Cray T3E node hours.

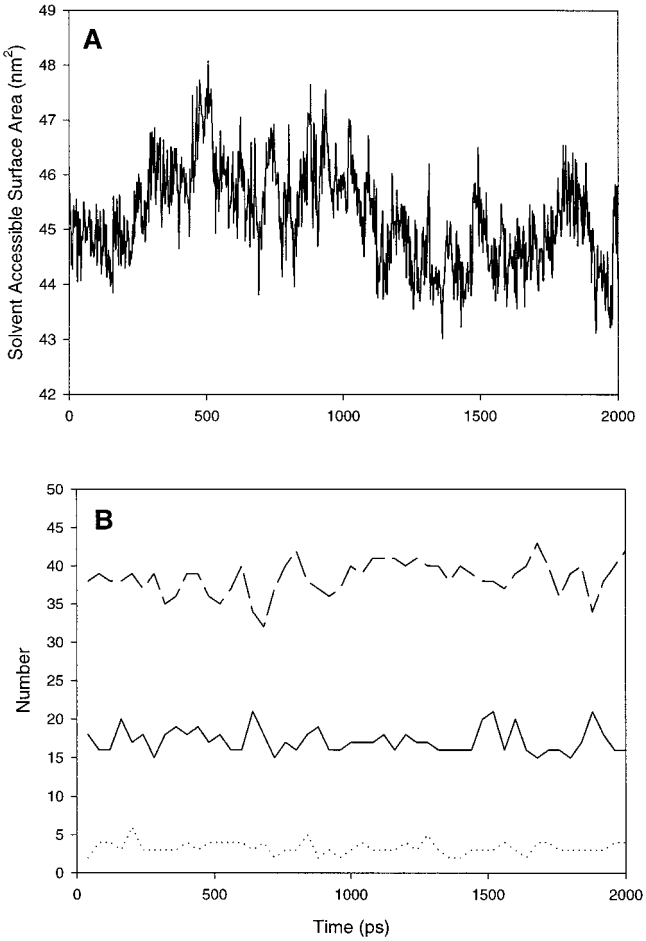

Fig. 2. Solvent-accessible surface area of the FAS2 molecule is shown in **A. B** shows the number of residues in random coil conformation (solid line), backbone hydrogen bonds (dashed line), and strained backbone dihedrals (dotted line).

RESULTS

Although an initial period of equilibration was provided, many systems require more than 25 ps to relax to the simulation conditions. Therefore, some initial portion of the molecular dynamics trajectory is often considered as an additional equilibration period and not used in the analysis of the simulation. How much of the trajectory to regard as equilibration steps and subsequently discard was decided using several sources of data. First, the solute potential energy showed a systematic decrease over the first 50 ps of the simulation. Such an initial decrease is often indicative of an equilibrative stage of the molecular dynamics simulation. Furthermore, the solvent-accessible surface area decreased systematically for the first 100 ps of simulation, indicating a longer period of equilibration than suggested by the solute potential energy. Due to these observations, the first 106 ps of simulation were discarded. While this choice may have been somewhat conservative, it allows for greater confidence in the results. Further discussion will refer to the remaining 2,074 ps of the trajectory.

Analysis of the various energetic components and thermodynamic properties is helpful in assessing the validity and performance of a MD simulation. The solute and solvent temperatures remained drift-free with small fluctuations, as expected since the simulation was coupled to a heat bath. The components of the solute energy can also provide insight into the state of the fasciculin-2 molecule during the simulation. Features such as drifts and jumps in the various energy components over the course of the trajectory could suggest conformational changes requiring further investigation. However, both the solvent and solute energies were free from such features, providing additional support for the stability of the present simulation.

A final indication of the simulation stability is in structural properties such as the number of residues in random coil conformation, solvent-accessible surface area, number of backbone hydrogen bonds, and number of strained backbone dihedral angles. These features were probed used the WHAT IF software package.[17] Figure 2 shows the behavior of these variables over the course of the simula-

tion. The solvent-accessible surface area shows a mean value of 45 ± 1 nm^2 with no significant drift. On average, less than one third (17 ± 2) of the 61 fasciculin-2 residues assume random coil conformation over the 2 ns of observation. The number of backbone hydrogen bonds fluctuates around a mean of 38 ± 2 and less than 3% (3 ± 1) of the backbone dihedral angles are in strained conformations.

The above energetic and structural evidence strongly suggests a stable MD trajectory; all of the temperatures and energetic properties showed negligible drift and reasonable fluctuations. Furthermore, Figure 2 shows that the structural properties are similarly well-behaved throughout the simulation. The absence of a positive drift in properties such as surface area suggests the protein remains in a compact globular form throughout the simulation. Likewise, the lack of a significant change in the number of backbone hydrogen bonds indicates the protein maintains roughly the same fold during the simulation. Finally, the consistently small number of strained dihedral angles suggests the protein sample's relatively energetically favorable conformations during the MD simulation.

In Figure 3A, the root mean square deviation (RMSD) of the protein (without ions) is plotted with respect to the FAS2 structure after the initial equilibration period (i.e., at 0 ps). As this figure illustrates, the RMSD shows a quick initial increase followed by a slow rise and finally a slight decrease throughout the remaining simulation. Figure 3B shows the relative mean square fluctuation for each atom and in the protein as well as the relative mean square fluctuations of the amide backbone nitrogens. These are scaled by the maximum values 0.06 and 0.01 nm^2 and the minimum values of 5×10^{-4} and 1×10^{-3} nm^2 for all the protein atoms and only the backbone nitrogens, respectively. The grouping of peaks and valleys in the solid curve of Figure 3B indicates the motion of side-chain and backbone atoms, respectively. Fluctuations of large magnitude occurred in atoms between 156–168, corresponding to the side chain of Arg 11 in the fasciculin-2 molecule. Smaller fluctuations were also observed in the side chains of Lys 25 (atoms 372–380), Arg 27 (atoms 403–415), Arg 28 (atoms 433–439), Pro 30 (atoms 462–469), Lys 32 (atoms 498–506), and Leu 35 (atoms 551–558). Backbone motions are also shown in Figure 3B and are concentrated near residues 7–12, 30–31, and 43–46. All three of these regions correspond to turns near the tips of the FAS2 loops. Additional backbone motion was observed for residues 16–20 and 54–57, which correspond to turn regions near the core of the FAS2 protein.

Principal component analysis (PCA), or "essential dynamics," diagonalizes the covariance matrix of atomic displacements to obtain a set of eigenvectors representing the motion of the protein through the dynamics trajectory.[24] The corresponding eigenvalues provide a measure for the magnitude of the atomic displacements represented by each eigenvector. Only a small fraction of the eigenvectors are represented by non-negligible eigenvalues. Since the eigenvectors are generally ranked by decreasing eigenvalue, the first few principal modes of motion for the trajectory contain most of the atomic fluctuations of the protein. Because of this, PCA is a useful technique for filtering large scale motions from molecular dynamics simulations.

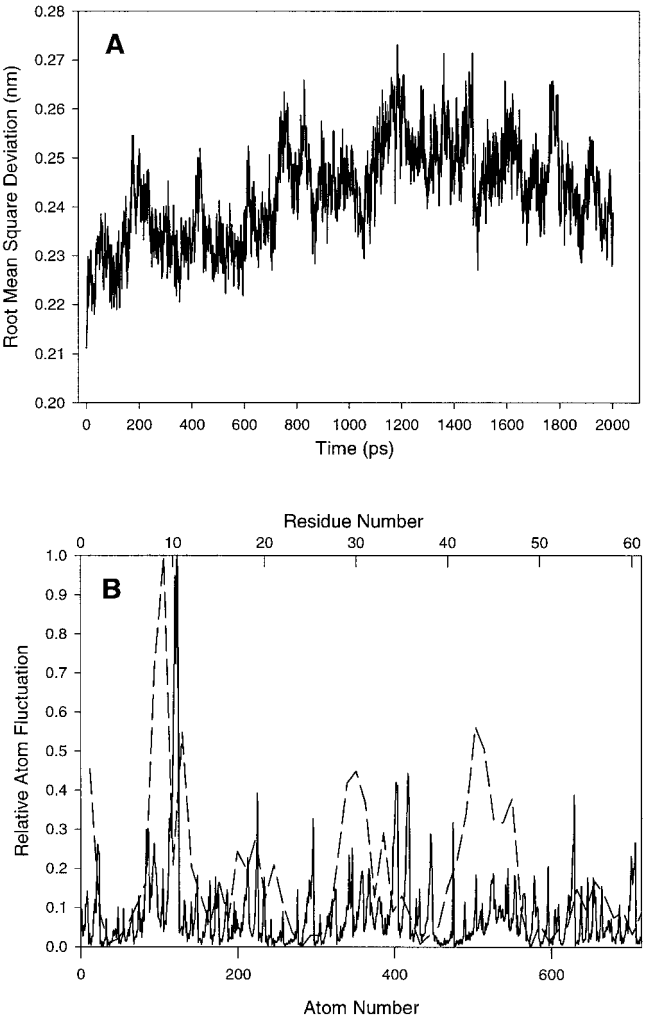

Fig. 3. The root mean square deviation (RMSD) from the starting FAS2 structure is shown in **A. B** depicts the relative fluctuations of all atoms in the FAS2 molecule (solid line, bottom axis) and the amide nitrogens of the FAS2 backbone (dashed line, top axis).

The covariance matrix for the FAS2 α carbons was constructed and diagonalized using WHAT IF. Figure 4A shows the distribution of eigenvalues. As expected, most of the atomic displacements were contained in the first few eigenvectors. The contributions of the α carbons to the first three eigenvectors are depicted in Figure 4B. This plot gives an estimate of the relative displacement of each residue due to the motion represented by this eigenvector. Figure 5 shows the distributions from projection of the MD trajectory along the first, third, and fifth largest eigenvectors. Note that the distributions of these projections become increasingly smooth; while the first and third eigenvectors have irregular distribution shapes, the fifth eigenvector projection is roughly Gaussian.

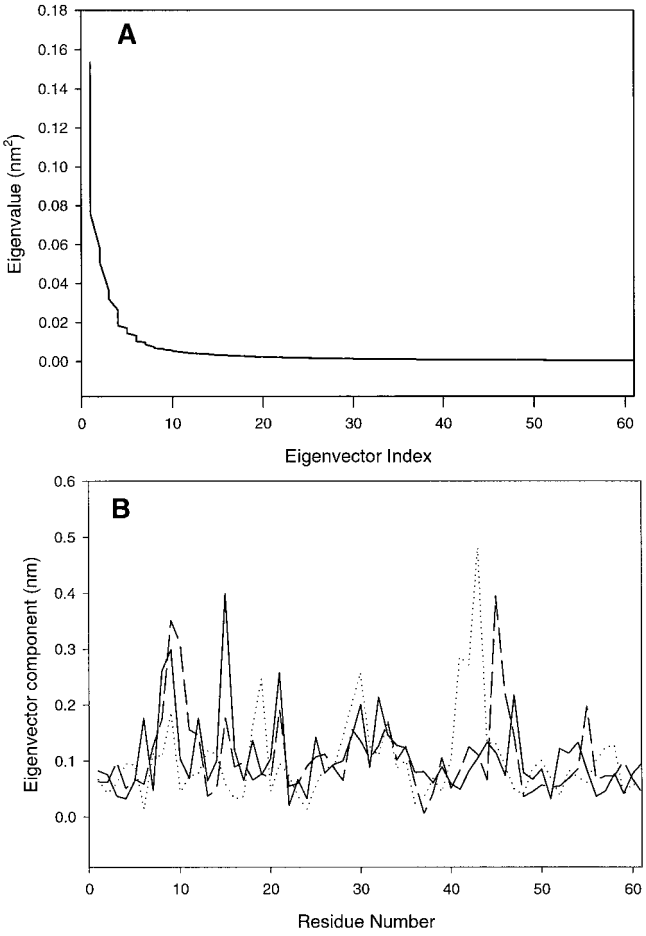

Fig. 4. **A** shows the magnitudes of the eigenvalues in the PCA or essential dynamics analysis (see text). The atomic components of the eigenvectors 1 (solid line), 2 (dashed line), and 3 (dotted line) are shown in **B.**

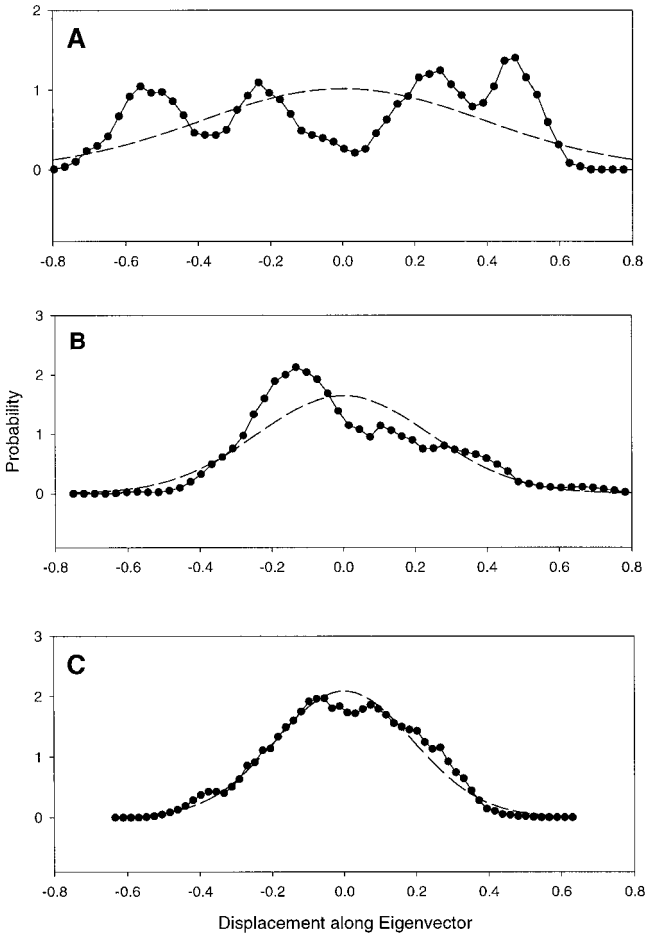

Fig. 5. Probability distributions along PCA eigenvectors (solid line with points) with corresponding Gaussian curve fit (dashed line). **A:** first eigenvector, **B:** third eigenvector, **C:** fifth eigenvector.

DISCUSSION

Ergodicity of the Molecular Dynamics Trajectory

The ergodic hypothesis states that over an infinite time interval, a system will sample all available states. In reality, this requirement is never completely fulfilled by finite simulations. However, some rough concept of the ergodicity of a MD trajectory is useful for determining the convergence of observables obtained from the simulation. Several properties of the simulated system can help estimate such sampling of a MD trajectory. As Figure 3 illustrates, the RMSD continued to rise through much of the simulation, indicating that the structure moved further from the starting structure as it explored configuration space. However, this is expected from the MD simulation and does not suggest further equilibration was necessary. The lack of convergence in the RMSD indicates the system had not finished exploring phase space when the MD simulation was terminated.

The projection of the trajectory onto PCA eigenvectors can provide additional information about ergodicity by considering sampling along the different principal modes. Well-sampled modes should have a Gaussian profile of sampling, while undersampled ones should appear skewed with several peaks.[24] In general, the largest modes obtained from PCA of a MD simulation are not sampled in a Gaussian fashion on the timescale of the simulation. As shown in Figure 5, only the fifth eigenvector, which contains small atomic displacements, has a very Gaussian distribution. This provides more support for the non-ergodicity of the simulation. However, incomplete sampling is neither surprising nor detrimental to the results of the simulation. While non-ergodicity may prevent this information from describing all the structural fluctuations of the protein, it does provide a concise description of the dynamics of the trajectory on the timescale of the simulation.

Protein Motions and Connections With NMR Data

Much of the data from the MD simulation points to movement in the loop regions of FAS2 that are associated with conformational changes in FAS2 when complexed with AChE.[9] Figures 3B and 4B depict large fluctuations in the backbone atoms of residues 7–13 over the course of the

simulation. These movements can be associated with the flexibility in loop I required for the conformational change upon binding AChE.[9] Likewise, the movement of residues 27–34 and 39–47 is consistent with the conformational changes observed at the tips of FAS2 loops II and III in the AChE-FAS2 complex with respect to the free fasciculin structures.[9] The components of PCA eigenvectors shown in Figure 4B also show that the residues implicated in conformational changes upon binding are involved in much of the observed motion. The flexible regions of the FAS2 protein backbone are summarized by color in Figure 1. In addition to the backbone motion, Figure 3B indicates large motions in side chains of residues, such as Arg 11, Pro 30, and Lys 32, also involved in FAS2 binding to AChE.[9] Additionally, other Lys, Arg, Pro, and Leu residues were observed to be especially mobile during the simulation. Such flexibility may be important for adjustment of side chains, especially those with positive charges, as FAS2 binds to the negative surface of AChE.

The molecular dynamics trajectory was visualized with the Insight II software package.[25] As suggested by the energetic and structural data, the protein was observed to remain very stable throughout the simulation. The ions drifted through the solvent box over the course of the simulation, sometimes associating with the protein, but often remaining free in solution. The β-sheets of the "finger" loops showed little variation, although the turns at the "fingertips" underwent considerable motion. The core region of fasciculin-2 also stayed rather rigid, with the majority of the motion again confined to the turn regions. Despite the high structural identity with FAS1 and the removal of the detergent molecule implicated in conformational differences between the FAS2 and FAS1 crystal structures,[7] loop I of FAS2 never adopted the conformation from the FAS1 structure. Instead it remained in a conformation close to the starting crystal structure of the free FAS2 molecule. As mentioned previously, this conformation is also similar to the position of loop I in the FAS2-AChE complex. This suggests that this "extended" conformation for loop I of fasciculin proteins is not entirely unstable and that it may vary with a relaxation time longer than the 2 ns of MD simulation reported here.

This hypothesis is supported by the NMR studies of Guenneugues et al. on the related three-finger protein, α toxin.[26] The NMR experiment reported much more microsecond-scale motion for the loop I region than for loops II or III.[26] Specifically, the turn region connecting loops I and II, the disulfide bond bridging these loops, and residues near the tip of loop I were all implicated in the motion. This suggests larger-scale motions at longer timescales that may facilitate the transition of loop I from a conformation observed throughout this simulation to a conformation similar to the FAS1 structure.[12] Although α toxin has only 45% sequence identity with FAS2, they share the similar structural characteristics of the three-finger toxin family. Therefore, the results of the NMR experiments are expected to be at least qualitatively applicable to the FAS2 system considered here.

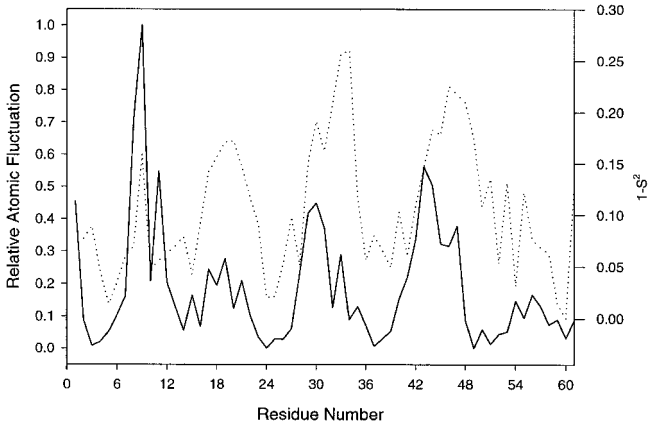

Fig. 6. Comparison of relative values of NMR amide nitrogen $1 - S^2$ for α toxin (dotted line, right axis) and relative values of the amide nitrogen fluctuations for FAS2 MD simulation (solid line, left axis).

The NMR study of Guenneugues et al. also examined the nanosecond timescale dynamics of α toxin.[26] The order parameter S^2 provides a measure of the relative rigidity of a portion of a molecule based on its anisotropic relaxation. The NMR experiments found high S^2 values for 70–80% of the backbone nitrogens in loops I–III, implying these portions of the backbone are nearly rigid on the nanosecond timescale.[26] Most of these rigid residues were found in the β-sheet region of the loop structures. Figure 6 shows the amide nitrogen atomic fluctuations data (from Fig. 3) plotted with the $1 - S^2$ values (thereby representing relative flexibility rather than rigidity) from the α toxin NMR study. This graph is in good qualitative agreement with the NMR data. For example, one strand of the β-sheet on loop I is defined by residues 2–8. As the NMR data suggests, these residues show smaller atomic fluctuations, indicating relative rigidity. Other regions of rigidity from the simulation data (residues 12–15, 21–27, 36–39, 47–51) are in also in agreement with the NMR data.

In addition to finding rigid regions of the toxin in agreement with the NMR data, the molecular dynamics data also identifies similar flexible regions as found in by Guenneugues et al.[26] However, for α toxin, loop II exhibited the largest deviations in its tip position, whereas the MD simulation of FAS2 shows the most motion in loop I. Both data show flexibility at the tips of the other loops, as well as in the turn connecting loops I and II. While the magnitudes differ somewhat and peaks are shifted by a few residues, these differences can be largely attributed to sequence differences between the two toxins. In general, the NMR and MD data are in good qualitative agreement.

CONCLUSIONS

The major regions of flexibility observed by MD on the nanosecond timescale include the regions of the protein implicated in binding to AChE. The tips of loops I and II were among the most flexible of these regions, with large motions in both the protein backbone and side chains of residues such as Arg 11 and Lys 32. This suggests that consideration of FAS2 flexibility may be important for

accurate modeling of FAS2 binding to AChE, implying that simple rigid body simulations might not be sufficient to account for these interactions. All of the flexible portions of the FAS2 molecule found by this simulation are in qualitative agreement with NMR studies of α toxin on the same timescale.

The MD simulation of FAS2 showed very little motion of loop I on a nanosecond time scale that could be attributed to a conformational change from its position in free FAS2 and FAS2-AChE crystal structures to the conformation observed in the X-ray structure of FAS1. It is possible that this motion occurs on much longer timescales. Motions of loop I in the related α toxin peptide have been observed by NMR on microsecond timescales.

The results of this work and related MD studies of AChE[27] will be important in motivating further simulations of AChE and FAS2 encounter where AChE and FAS2 are allowed some internal degrees of freedom. Previous studies involving protein-protein encounters have primarily dealt with rigid proteins.[28] Although additional simulations may be required to elucidate the longer timescale motions of these proteins, the simple motions uncovered by this simulation could allow for a simple representation of important motions of FAS2 and AChE during encounter simulations.

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