Direct Simulation of Transmembrane Helix Association: Role of Asparagines

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ABSTRACT The forces contributing to the association of transmembrane helices in folded membrane proteins have received considerable attention recently. In this study we investigate the importance of hydrogen bonding by studying the effect of a single Asn residue in the center of an otherwise hydrophobic transmembrane peptide using computer simulations. We use the model peptide MS1 which has been derived from the leucine zipper coiled-coil dimer of the transcription factor peptide GCN4-P1. We follow the trajectory of 36 initially monomeric MS1 transmembrane helical peptides in a membrane-mimicking octane layer as they associate into larger structures. These peptides predominately form dimers. The interaction between the polar asparagine residues, capable of simultaneously being a hydrogen-bond donor and acceptor, contributes strongly to the stability of associated helices. Only dimers with interhelical hydrogen bonds form stable structures, whereas aggregates without any hydrogen-bonding interactions form very transient structures. We examine the hydrogen-bonding patterns and find that there are two forms of dimer, one with symmetric hydrogen bonds and one with asymmetric hydrogen bonds. Based on the structures in our simulation we propose a model with a monomer ↔ symmetric dimer ↔ asymmetric dimer ↔ trimer equilibrium.

INTRODUCTION

Membrane proteins probably represent more than a quarter of all proteins encoded in genomes (Liu and Rost, 2001). They play an important role in cell biology and physiology, carrying out many essential cell functions. The forces stabilizing membrane protein structures have received considerable attention, although detailed structural information is difficult to obtain. A two-state model for membrane protein folding proposes that folding occurs in two separate steps (Popot and Engelman, 1990). The first step involves insertion of the transmembrane helices; the second involves the formation of specific interactions between these helices, forming tightly packed native structures. The association of TM helices can occur as either an inter- or intramolecular process. For example, bacteriorhodopsin (Kataoka et al., 1992) has been shown to fold into a functional protein by refolding from peptides representing its transmembrane helices, and many membrane proteins consist of several subunits which must associate to form the fully functional protein. Known motifs of closely packed helices in membranes are e.g. the GxxxG or the heptad repeat motif. In the GxxxG packing motif (Rees et al., 1989; Fleming and Engelman, 2001) the close approach of the backbones at the glycine positions facilitates weak hydrogen bonds between α-carbon hydrogens and carbonyl groups stabilizing the dimer structure. In contrast, the soluble GCN4-P1 peptide has been found to form a left-handed coiled-coil structure (Crick, 1953; O'Shea et al., 1991) associating with a heptad repeat motif (Bowie, 1997; Walther et al., 1998) as shown in Fig. 1. In the heptad repeat motif the number of residues per turn is reduced to 3.5 compared to the normal 3.6 residues per turn, thereby improving the packing of the coiled-coil structures and repeating the packing motif each seven residues. The side chains of each residue of the dimer interface protrude into cavities formed by four side chains of the neighboring helices in a regular manner termed knobs into holes or leucine zipper packing (Langosch and Heringa, 1998; Zhou et al., 2001).

Van der Waal's interactions and effective packing of hydrophobic residues have been identified to play an important role in the stability of membrane proteins (White and Wimley, 1999; Popot and Engelman, 2000). Other possible important interactions include electrostatic effects, hydrogen-bond formation, prosthetic group interactions, side-chain entropies, and lipid-protein interactions. Buried polar residues are relatively rare in polytopic TM proteins, but are highly conserved (Lear et al., 2003), suggesting either a functional or a structural role (Jones et al., 1994; Arkin and Brunger, 1998). Experiments (Gratkowski et al., 2001; Zhou et al., 2001) have shown that a number of polar side chains can mediate the association of TM helices, most probably through interhelical hydrogen bonds. In particular, the residues that are capable of simultaneously being hydrogen-bond donors and acceptors (Asn, Asp, Gln, and Glu) mediate oligomerization of helices in biological membranes and micelles (Choma et al., 2000; Zhou et al., 2000, 2001; Gratkowski et al., 2001). The free energy contribution ($\Delta\Delta G$) of these polar residues relative to nonpolar amino acids is in the range of 1–2 kcal/mol (Gratkowski et al., 2001).

Polar residues such as Asn and Gln are not equally distributed over the surface of known TM helices, but exhibit

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Abbreviations used: GCN4-P1, peptide from yeast transcription factor GCN4; MS1, model transmembrane peptide based on GCN4-P1; TM, transmembrane; DPC, dodecyl phosphocholine.

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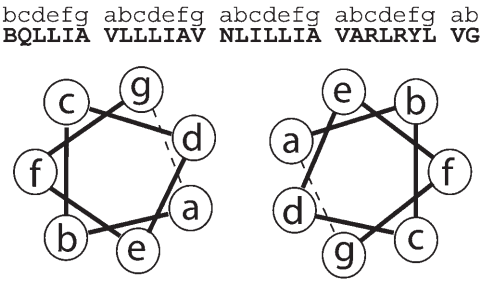

FIGURE 1 Heptad repeat of the coiled-coil structures. The dimer interface consists of residues in the *a* and *d* positions. The polar Asn residue of MS1 occupies an *a* position in the dimer interface. The sequence of the MS1 peptide is shown together with its heptad repeat positions.

a strong tendency to be buried in the interface in the middle of the bilayer (Lear et al., 2003). If not buried within the protein structure these residues could potentially lead to nonspecific interactions between membrane proteins leading to misfolding and misfunction (Bowie, 2000; Zhou et al., 2000). There are several examples of nonpolar to hydrogen bonding residues leading to disease including the single mutation (V664E) in the neu/erb-2 protooncogene (Bargmann et al., 1986; Smith et al., 1996) leading to constitutive activation of the encoded tyrosine kinase receptor, or the V232D mutation in the TM4 helix of cystic fibrosis transmembrane conductance regulator (Partridge et al., 2002) altering the structure and function of the mature protein. Two Asn mutants (M701N and G708N) of the transmembrane helix $\beta 3$ of integrin αIIb$\beta 3$ have been identified to drive the association by homooligomerization (Li et al., 2003) activating the mutant integrin to constitutively bind fibrinogen.

In this study we are investigating the association behavior of single-folded transmembrane helices into higher aggregates and the importance of polar residues in the center of the membrane for specificity and stability using the model peptide MS1, designed by Choma et al. (2000). MS1 is based on a hydrophobic version of the leucine-zipper GCN4-P1 peptide, a 32-residue long homodimeric coiled-coil peptide from the yeast transcription factor GCN4 (O'Shea et al., 1991). The soluble parent peptide GCN4-P1 forms coiled-coil dimers, whereas mutants have been shown to exist in different association states (monomers, dimers, and trimers) (O'Shea et al., 1991; Harbury et al., 1993; Gonzalez et al., 1996a,b; Lino et al., 1996). The membrane soluble MS1 peptide conserves the seven residue heptad repeat (Langosch and Heringa, 1998; Gurezka et al., 1999) of GCN4-P1, with Leu residues occupying the ''d'' positions, and Val residues at all but one ''a'' position. The hydrophobicity of the interface is interrupted at the central ''a'' position by an Asn residue that is capable of forming hydrogen bonds across the dimer interface. MS1 associates in micelles in a reversible monomer-dimer-trimer equilibrium. This equilibrium depends on the type of micelle (Gratkowski et al., 2002) with a larger degree of trimers in C14-betaine and SDS micelles than in DPC micelles.

We are using MD simulations to investigate the association behavior of MS1 by following the trajectory of 36 peptides in a model membrane system over time. The dominant association state present in our simulation is the dimeric form, stabilized by interchain hydrogen bonds between the carboxamido groups of the Asn side chains. These dimer structures are on average left-handed coiled-coil structures indicating that the membrane soluble MS1 peptide assumes indeed a coiled-coil structure comparable to GCN4-P1 in water.

METHODS

Simulation setup

The starting structure was produced by inserting 36 identical, ideal α-helices built from the sequence of MS1 (BQLLI AVLLL IAVNL ILLIA VARLR YLVG, B=β-Ala) on a regular grid into a 3.0 nm thick layer of octane. The entire system was then solvated, and sodium and chloride ions were added to give a charge neutral system with a salt concentration of ~250 mM. The final simulation box measured 17.0 × 17.0 × 7.5 nm and contained 3024 octane molecules, 37,764 water molecules, 180 Na^+, and 288 Cl^- ions.

Molecular dynamics simulations were carried out using the GROMACS 3.0 MD package (Berendsen et al., 1995; Lindahl et al., 2001) applying periodic boundary conditions. The peptide and octane were represented using the GROMOS96 43a2 force field (Scott et al., 1999), whereas water was represented using the SPC model (Berendsen et al., 1981). The simulation was carried out for 45 ns with the temperature maintained at 300 K using a Berendsen ($\tau_T = 0.1$ ps) thermostat (Berendsen et al., 1984), coupling the protein, the octane, and the water/ions separately. The area of the octane/water interface was held fixed and the pressure in the direction normal to the interface was maintained at 1 bar using the weak coupling algorithm (Berendsen et al., 1984) with a coupling constant of 1.0 ps and a compressibility of 4.6×10^{-5} bar^{-1}. The electrostatic interactions were evaluated using the smooth particle mesh Ewald method (Darden et al., 1993; Essmann et al., 1995), with a real-space cutoff of 0.9 nm. The long-range electrostatic interactions were calculated with fourth-order B-spline interpolation and a Fourier spacing of 0.16 nm. The Lennard-Jones interactions were evaluated using a twin-range cutoff (0.9 and 1.4 nm) with the neighbor list updated every five steps. All bonds in the peptides and octane were constrained using LINCS (Hess et al., 1997). The bonds and angles of water were constrained using the SETTLE algorithm (Miyamoto and Kollman, 1992). Additionally, the hydrogen atoms of the peptides were treated as dummy atoms (Feenstra et al., 1999) allowing a time step of 5 fs.

Model membrane system

The octane layer used in our simulation models the aliphatic region of the lipids in the bilayer as it has approximately the same thickness, whereas the headgroup region is not included in our model. The use of octane as a membrane-mimicking environment enables us to investigate the association process and observe the formation of dimers, trimers, and higher-order aggregates. The lateral diffusion properties of octane allow single peptides to come into contact within a few nanoseconds, whereas a full representation of the lipid environment would make the association almost impossible to simulate because of a much lower lateral diffusion coefficient, slowing down the diffusion and therefore shifting the timescale of the association process far beyond the time window accessible by simulations.

The starting configuration of our simulations is shown in Fig. 2 *A*, with the 36 peptides equally distributed over the simulation box. The peptide/octane ratio is 1:84, which is roughly equivalent to a peptide/DPC ratio of ~1:56 (on the basis of the lipid hydrophobic tail). This concentration is only ~3–6 times larger than the concentration used by Gratkowski et al. in their experiments, and allows us to follow association events in a reasonable time

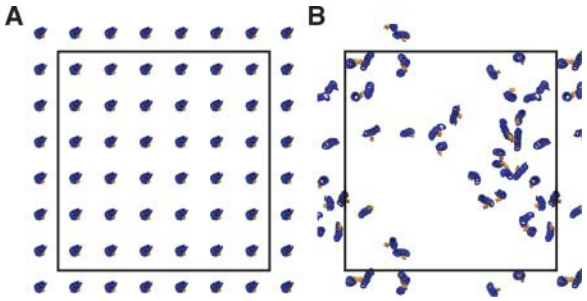

FIGURE 2 Snapshots of the trajectory (core region of peptides only) showing the starting structure (*A*) and the final structure (*B*) from our simulation. The MS1 helices are shown in blue, the Asn residues are shown in orange, and the rectangle represents the simulation box. The periodic images are added for clarity. The final structure is a snapshot of a very dynamic and transient system with nine stable dimers whereas all other close helices are rapidly diffusing.

window obtaining results that are close to the experimental data in DPC (Gratkowski et al., 2002).

Analysis

Our analysis indicated that the association properties of MS1 are governed by the interactions of the residues of the hydrophobic stretch of the MS1 peptide comprising residues 3 (Leu) through 22 (Ala) inserted in the membrane-mimicking octane layer. Therefore the analyses are done on this core region if not stated otherwise. Crossing angles were calculated using the TWISTER algorithm (Strelkov and Burkhard, 2002). Hydrogen bonding structures were determined by clustering pairwise combinations of Asn carboxamido side-chain atoms, using an RMSD-based clustering algorithm (Daura et al., 1999) with a cutoff of 0.04 nm. The association state of MS1 was determined by measuring atom-atom distances. Two peptides were defined as being in contact, if any atom-atom distance d_{ij} between two peptides are below a cutoff of 0.44 nm, which is very near the minimum of the CH_2-CH_2 Lennard-Jones function used in this study.

RESULTS

Structure of associated peptides

The initial aggregation process of MS1 (Fig. 3) is characterized by fast formation of transient aggregates. Contacts between peptides can be identified after the first nanosecond with their numbers increasing over the entire 45 ns trajectory. The majority of these contact events do not lead to the formation of stable structures, but are transient, disassociating on a picosecond timescale, and involve only a few atoms on each helix. The average number of dimers with at least two atoms in van der Waal's contact increases with simulation time as shown in Fig. 3, reaching a final molecular fraction near fifty percent with the dimeric state being the dominant association form (Fig. 3 *A middle panel*). The inclusion of all atoms of each peptide chain shows a higher aggregation (Fig. 3 *A*, *upper panel*) rate that is not representative for the real fraction of associated peptides, and likely related to the limitations of our model membrane system that does not include the lipid headgroups. The fraction of close contacts of

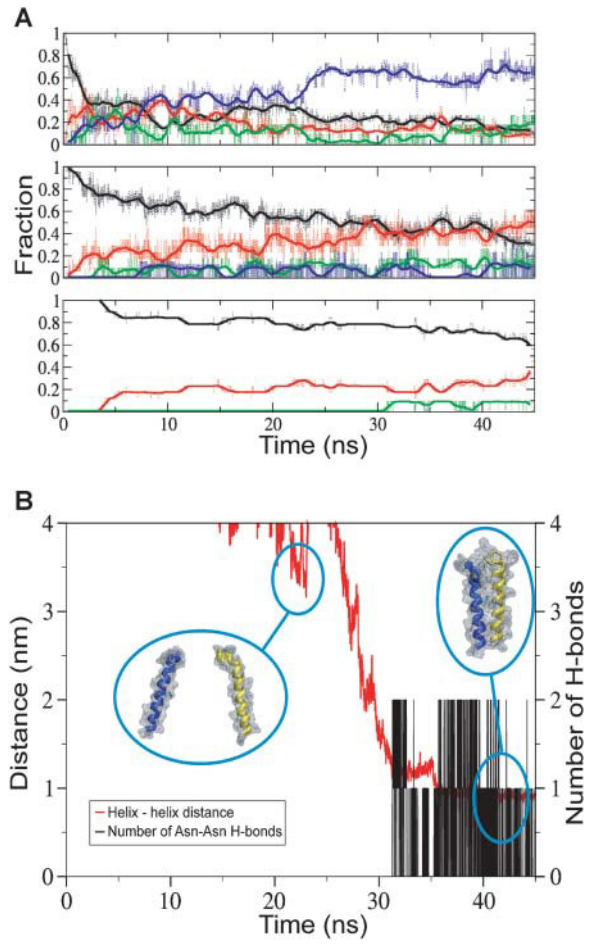

FIGURE 3 Association state distribution of MS1 peptides (*A*) listed as monomeric (*black*), dimeric (*red*), trimeric (*green*), and tetrameric and larger (*blue*) focusing on the complete length of the peptides (*upper panel*), the hydrophobic core region (*middle panel*), and the Asn residue inside the membrane (*lower panel*). Dimer formation (*B*) showing the number of hydrogen bonds (*black*) between the Asn residues, and the center of mass distance (*red*) of the two helices of a dimer with the actual dimer structures displayed in the inserts. The Asn residues are highlighted in orange.

the Asn residue correlates well with the fraction of close contacts between peptides indicating that a large percentage of the associates are stabilized by the formation of interchain hydrogen bonds (see Fig. 3 *A, lower panel*). The final structure of our 45 ns trajectory is shown in Fig. 2 *B*. This figure represents a snapshot of a highly dynamic system with transiently associated aggregates and it contains a number of very stable hydrogen-bonded dimers.

These loose aggregates occasionally associate into tightly packed stable structures. The formation of these well-packed structures, compared to the formation of transient peptide-peptide contacts, is a much less frequent process, recognized by the following criteria over a ns time period: a lower interhelical distance (center of mass distance: 0.9–1.3 nm), a smaller range of helix-helix center of mass distance fluctuations compared to helices transiently associated, a much

greater structural stability, and a large number of van der Waal's contacts all along the TM helix. Almost all of these stable dimers show hydrogen bonds across the dimer interface, consisting almost entirely of the peptide fraction of Asn-mediated hydrogen-bonded dimers (Fig. 3 *A*, *lower panel*).

Only one hydrogen-bonded and tightly packed trimer formed during the 45 ns time window. This symmetric trimer developed from a dimer by the inclusion of a third peptide. The stability of this trimeric complex seems to be lower than that of dimers, as after several ns this complex changed to a more open structure and converted into two dimers with the involvement of a fourth peptide. A longer simulation would be required to show the formation of more of these trimeric structures allowing a more thorough analysis.

Dimer conformations

In our simulations we see a clear tendency toward the formation of left-handed coiled-coil structures over time. At the moment of dimer formation we find a slightly higher number of right-handed structures. Over time the amount of left-handed dimers increases at the expense of the right-handed conformation. The left-handed coiled-coil dimers are highly stable, and once formed these complexes generally remain associated for the remaining simulation time. The right-handed coiled-coil structures are less stable, as two of these initially right-handed coiled-coils change their conformation—making a transition toward a left-handed coiled-coil structure without disrupting the hydrogen bonds between the Asn residues as shown in Fig. 4. No transition from a left-handed toward a right-handed coiled-coil could be identified. At the endpoint of our simulation 5 left-handed coiled-coils and only three right-handed can be observed.

A very low number of tightly packed dimers could be identified that do not involve any interhelical hydrogen bonds. Most of them are barely stable, disassociating in a subnanosecond time range, but a few form tightly packed dimers that are stable for several nanoseconds. Interestingly the interhelical distance of these structures is generally smaller compared to the dominantly formed dimers, indicating a better overall packing and stronger van der Waal's interactions.

Types of interhelical hydrogen bonds

The Asn-mediated dimers are not restricted to one single conformation. Several conformations with hydrogen bonds across the dimer interface can be identified, with all keeping close packing and many hydrophobic interactions over the entire dimer interface (Fig. 4). The interhelical hydrogen bonding pattern differs between single dimers: i), the main group of dimeric structures is characterized by hydrogen bonds across the dimer interface involving only Asn side chains, discussed below; and ii), a second group of dimers is

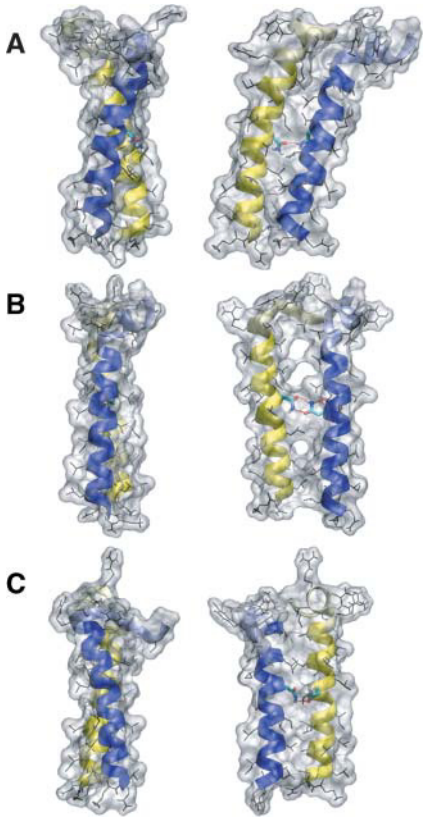

FIGURE 4 Snapshots showing a transition from the right- to a left-handed conformation. (*A*) Snapshot at 20 ns showing a right-handed crossing angle. (*B*) Snapshot at 40 ns showing an approximate parallel orientation. (*C*) Snapshot at 45 ns showing a left-handed coiled-coil structure. The Asn residues are highlighted in orange.

characterized by interhelical hydrogen bonds between one Asn side chain and the backbone of the second helix. The stability of these structures is lower compared to the previously described structures and these dimers are less frequent. The hydrogen bonds are much weaker, as the N-O distances are generally large, the hydrogen bonding angle is not ideal and because this type of interchain hydrogen bond disturbs the helical backbone hydrogen bonds. Additionally the inherent asymmetry leaves the Asn of the second peptide chain without stabilizing interactions.

Interhelical Asn mediated hydrogen bonds

We can identify two distinct populations of interhelical hydrogen-bond patterns of dimers as displayed in Fig. 5. Both species are very stable in the time window of our simulation showing hardly any interconversion. The first group consists of structures that are characterized by only one hydrogen bond between the two Asn side chains in line with the asymmetric hydrogen-bond described in the crystal structure of GCN4-P1 (O'Shea et al., 1991). The other potential hydrogen bond donor (amide group) or acceptor (carbonyl oxygen) is occasionally involved in a hydrogen

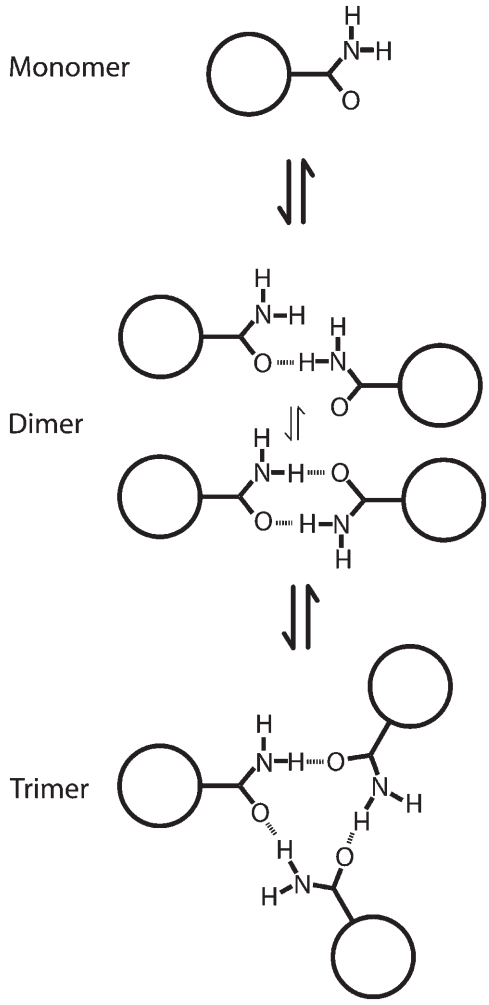

FIGURE 5 Model describing the monomer, dimer, and trimer structures and the interhelical hydrogen-bonding pattern present in our simulations.

bond to its own backbone or in an interchain hydrogen bond to the backbone of the second chain. The second dimeric form is a more symmetric structure with two interhelical hydrogen bonds across the dimer interface between the side chains of the Asn residues. The formation of these two hydrogen bonds permits both amide and carbonyl groups to be involved in one hydrogen bond each.

DISCUSSION

The measured difference in association states of the MS1 peptide (and related mutants) between SDS, DPC, and C14-betaine suggest that the equilibrium association states are remarkably influenced by the environment, with MS1 being dominantly dimeric in DPC micelles, while forming mostly trimers in C14-betaine and SDS micelles (Gratkowski et al., 2002). We expect therefore only moderate agreement between our average properties and the experimental data of MS1 in micelles. Our trajectory indicates that MS1 has a clear tendency to form dimer (~50%), but lower for trimers (~15%) and higher aggregates (~10%), although our system has not yet reached equilibrium after 45 ns. In DPC micelles (Gratkowski et al., 2002) a relative distribution of ~10% monomer, 70% dimer, and 20% trimer would be expected with a comparable lipid/peptide ratio.

The most common feature of the associated MS1 peptides is the Asn-mediated dimerization with many van der Waal's contacts along the remainder of the helices and some interaction between N-terminal amphipathic regions outside the octane slab. The polar Asn residue, situated in the dimer interface in the middle of the membrane spanning stretch, was identified by experiments to be crucial for the stability of MS1 oligomers. Indeed mutating this polar residue to Val, removing the only polar residue, largely abolished the association properties of MS1. In our simulations the same polar residue has been found to be important for stability. A clear correlation can be identified between the fraction of close contacts of the Asn residues and the presence of dimers and trimers (Fig. 3 *A, middle* and *lower panel*), outlining the important role this polar residue is playing in the association. These interhelical hydrogen bonds are a strong structural feature, determining stability with an additional contribution from van der Waal's contacts. The property of the Asn residue to be at the same time hydrogen-bonding donor and acceptor seems to be determinant for the structure-stabilizing effect, with the strength of a hydrogen bond expected to be large inside the hydrophobic environment of the bilayer.

The conformations of the side chains of this Asn residue have been investigated by NMR experiments by Zhou et al. (2000) using a GCN4-based leucine zipper peptide with identical residues in the dimer interface (''a'' and ''d'' position) and Leu residues at all other positions. In micelles at least two distinguishable populations of the side chain amide protons of the Asn residue can be identified that slightly differ in their chemical shift as a result of differences in their local chemical environment on the NMR timescale. The Asn side chain shows fast rotations in our simulation, suggesting an averaging effect. Therefore smaller differences will probably not be resolved in the NMR spectra. The effect of the hydrogen bonding on the chemical shift should nevertheless be visible in an NMR spectrum in the micellar environment and could give rise to the two populations observed by Zhou et al. (2000), because hydrogen bonds have a large effect on the chemical shift and the rotation of the Asn side chains does not affect the stability of these hydrogen bonds that remain intact. Based on our simulations, we postulate a model of the monomer, dimer, and trimer structures and the hydrogen-bonding interactions of MS1 (shown in Fig. 5) that is similar to the model proposed by Zhou et al. (2000), the difference being that we suggest the existence of two different stable species of dimers. We propose that the monomeric MS1 peptides associate into two types of dimers, both relatively stable. One is a symmetric structure with two hydrogen bonds across the dimer

interface, whereas the second conformation is characterized by only one hydrogen bond. The latter asymmetric structure is in line with the asymmetric hydrogen-bonding pattern described in the crystal structure of GCN4-P1. The relatively limited sampling of trimeric structures leaves some uncertainty on possible trimeric states. The trimer structure identified in our simulation is a very symmetric structure with the Asn residues in the middle of the helix bundle forming hydrogen bonds to the other peptides.

The formation of stable dimers without the involvement of hydrogen bonds is unexpected, as ultracentrifugation and SDS-PAGE experiments could not identify clearly detectable amounts of dimers in the N14V mutant (14), although showing traces of dimers in the case of the N14L mutation (Gratkowski et al., 2001). Our finding of these dimers are supported by results obtained by Gurezka et al. (1999; Gurezka and Langosch, 2001) who have shown that almost any combination of the hydrophobic residues LMIVF in the ''a'' and ''d'' positions of the heptad motif can form stable dimers in bacterial *Escherichia coli* membranes. Leu, Ile, and Val were particularly common in sequences that promoted dimer formation. Both these experimental data and our result suggest the presence of a low fraction of dimers stabilized by hydrophobic interactions without contributions from hydrogen bonds.

CONCLUSIONS

The conformation of MS1 has been expected to assume a left-handed coiled-coil structure like the parent water soluble GCN4-P1 structure. Although our data are not sufficient to determine the equilibrium distribution of structures, our simulations give a first indication of the structural preferences of MS1 as we can identify a clear tendency toward the formation of left-handed coiled-coils, confirming this prediction.

Hydrogen bonding between buried polar residues capable of being simultaneously hydrogen-bond donor and acceptor can clearly be identified to contribute dominantly to the stability of associated helices in membranes. In our simulations, only dimers that are held together by hydrogen bonds between the carboxamido group of the Asn side chains form very stable structures, whereas structures excluding such interactions are less stable, less frequent, and have short lifetimes. Hydrophobic interactions additionally contribute to the stability of tightly packed dimers, but do not seem to be determinant as seen from the time window of our simulations.

Two types of dimers with different interhelical hydrogen-bonding patterns, one symmetric, the other asymmetric, can be identified and both seem equally likely, suggesting a complex situation with two contemporaneously populated configurations with different binding modes.

The results of this study make us confident of the validity and usefulness of this large-scale approach to investigate the association behavior of membrane peptides. Further studies will investigate the equilibrium properties of MS1 and address the effect of single amino acid mutation.

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