The transmembrane homotrimer of ADAM 1 in model lipid bilayers

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Abstract

Fertilin is a transmembrane protein heterodimer formed by the two subunits fertilin α and fertilin β that plays an important role in sperm–egg fusion. Fertilin α and β are members of the ADAM family, and contain each one transmembrane α -helix, and are termed ADAM 1 and ADAM 2, respectively. ADAM 1 is the subunit that contains a putative fusion peptide, and we have explored the possibility that the transmembrane α -helical domain of ADAM 1 forms homotrimers, in common with other viral fusion proteins. Although this peptide was found to form various homooligomers in SDS, the infrared dichroic data obtained with the isotopically labeled peptide at specific positions is consistent with the presence of only one species in DMPC or POPC lipid bilayers. Comparison of the experimental orientational data with molecular dynamics simulations performed with sequence homologues of ADAM 1 show that the species present in lipid bilayers is only consistent with an evolutionarily conserved homotrimeric model for which we provide a backbone structure. These results support a model where ADAM 1 forms homotrimers as a step to create a fusion active intermediate.

Keywords: ADAM 1; homotrimer; fertilin; fusion; infrared dichroism; molecular dynamics; lipid bilayers; α -helical bundles

Fertilin is a transmembrane protein heterodimer that plays an important role in sperm–egg fusion. This heterodimer is located at the sperm surface, and is formed by the two subunits fertilin α and fertilin β . Fertilin α and β are members of the ADAM family (A Disintegrin and A Metalloprotease), and are referred to as ADAM 1 and ADAM 2, respectively (Becherer and Blobel 2003). The members of this family contain multiple domains: pro-domain, metalloprotease, disintegrin, cysteine-rich, EGF-like, transmembrane, and cytoplasmic tail. To date, about 40 ADAM proteins have been identified, according to the Table of the ADAMs, maintained by Dr. Judith White (University of Virginia School of Medicine; http://www.people.virginia.edu/~jw7g/Table_of_the_ADAMs.html). These proteins are expressed in a wide variety of somatic

tissues and half of them are expressed specifically, or predominantly, in the testis (Primakoff and Myles 2000).

Although not all ADAMs have metalloprotease activity, some ADAM proteins can act as proteases, through their metalloprotease domain, and also as cell adhesion proteins, through their disintegrin-like, EGF-like, and cysteine-rich domains. The best-studied cell adhesion activity of ADAMs is the interaction of sperm and egg during fertilization, and it has been suggested that ADAM 1, ADAM 2, and ADAM 3 are the three important players in this event by facilitating sperm–egg interaction (Cho et al. 1998; Kim et al. 2004; Nishimura et al. 2004). Indeed, some in vitro (Primakoff et al. 1987) and in vivo (Cho et al. 1998, 2000; Nishimura et al. 2004; Kim et al. 2006) studies suggest that ADAM 1 and ADAM 2 are required for efficient fertilization of the egg. The two latter proteins, in guinea pig and bovine sperm, have been shown to form heterodimers (Blobel et al. 1990; Waters and White 1997).

Both ADAM 1 and ADAM 2 are synthesized as full-length precursor proteins, which are proteolytically

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processed during spermatogenesis, leaving the disintegrin domain as N-terminal in the mature protein (Blobel et al. 1992; Wolfsberg et al. 1993). ADAM 1 is processed first in the testis, followed by ADAM 2 in the epididymis (Blobel et al. 1990). In mature sperm, both ADAM 1 and 2 are localized in the plasma membrane, restricted to the posterior head of the plasma membrane region (Hunnicut et al. 1997; Cowan et al. 2001). Analysis of ADAM 1 sequence reveal that the EGF-like repeat and the cysteine rich domain are important for sperm–egg binding, and there is an adhesion-mediating motif, DLEECDCG, in its disintegrin domain (Wong et al. 2001). In the mouse, ADAM 1, one of the ADAMs that has an active metalloprotease domain, is also expressed in other cell types, although at low levels: brain, heart, kidney, liver, lung, skeletal muscle, spleen, and ovary (Wolfsberg et al. 1995).

Whereas mature ADAM 2 retains its full-length disintegrin binding domain, ADAM 1 contains a putative fusion peptide which is relatively hydrophobic in its cysteine-rich domain, suggested to be an amphipathic α -helix (Blobel et al. 1992). The latter is reminiscent of viral fusion peptides, and supports a role for ADAM 1 in sperm–egg fusion. Indeed, this putative fusion peptide is able to fuse large unilamellar liposomes, particularly when using negatively charged lipids (Muga et al. 1994; Martin and Ruyschaert 1997; Martin et al. 1998).

However, apart from several studies that have characterized this putative fusion peptide (Muga et al. 1994; Martin et al. 1998; Wolfe et al. 1999), there is at present no structural information on ADAM 1. Also, its precise function, and specifically its role in fertilization is still unclear. For example, the current model for the mechanism of sperm–egg fusion, in which binding of heterodimeric ADAM 1/ADAM 2 to an unknown egg receptor is followed by a conformational change in ADAM 1, exposing the fusion peptide and promoting membrane fusion, is challenged by recent findings that show that sperm from ADAM 1 knock-out mice is still able to fuse with the egg (Kim et al. 2006). Furthermore, Izumo, a protein that belongs to the family of immunoglobulins, has been identified as a potent fusion protein located at the sperm surface (Inoue et al. 2005).

Each of the domains of ADAMs, except the transmembrane domain (TM), have been found to have a specific function either in proteolytic, signaling, adhesion, and fusion (Wolfsberg and White 1996; Primakoff and Myles 2000) processes. The TM domain however, may also have an important role. For example, being involved in TM heterodimeric or homomeric interactions critical for various functions.

As suggested previously (Doncel 2006), there are striking similarities between the α β fertilin system (ADAM 1 and ADAM 2) with viral fusion proteins,

e.g., the hemagglutinin subunits (HA1 and HA2) of Influenza (Skehel and Wiley 2000), or the envelope proteins gp120 and gp41 of HIV-1 (Wyatt and Sodroski 1998). These are also formed by two subunits, one being involved in binding and the other in fusion. The latter subunit forms homotrimers, either induced by low pH as in HA2 (Swalley et al. 2004) or after receptor binding of the partner binding subunit in gp41 (Weissenhorn et al. 1997), and this oligomerization is a key step in the fusion event. While these trimeric interactions have been detected in the ectodomain, they may also be present in the transmembrane domain.

By analogy, herein we test the hypothesis that ADAM 1, which also contains a fusion peptide, is able to form homotrimers, and that this interaction might take place at the transmembrane α -helical domain (ADAM1-TM). We have attacked this problem by synthesizing isotopically labeled ADAM1-TM and incorporating the peptide in model lipid bilayers. The size of the oligomer was obtained by comparing the orientational data of the peptide in lipid bilayers using polarized attenuated total reflection Fourier Transform infrared spectroscopy (PATIR-FTIR) with the predicted values in models obtained after a full conformational search using evolutionary conservation data (Briggs et al. 2001). Specifically, ADAM1-TM was synthesized introducing a labeled carbonyl ($^{13}\text{C}=^{18}\text{O}$) at two consecutive positions, and we determined the orientation in space of these labeled peptidic carbonyl groups using the theory of site specific infrared dichroism (SSID) (Arkin et al. 1997), which is especially suitable to study transmembrane α -helical bundles (Torres et al. 2000a,b, 2002). The results obtained add support to a trimeric conformation of ADAM 1 in fusion, by comparison to similar viral fusion proteins.

Results

Global search molecular dynamics

Simulations were performed using the homologous sequences shown in Figure 1, assuming different homooligomeric sizes, from dimers to hexamers. A common model, or more than one, was only found for trimers and tetramers. When fertilin α was assumed to be a homotrimer, two complete sets (Briggs et al. 2001; Torres et al. 2005) were found, and only when the helix tilt was restrained to 35° for a left-handed configuration. No other complete sets were found for other tilts or right-handed configurations. The RMSD between any pair of structures belonging to these complete sets was never higher than 1.5 Å. The helix tilt was the same for the two models found, 31° , whereas the rotational orientation ω for residue L14 was -77° and 178° , respectively, i.e., a difference of $>100^\circ$.

When fertilin α was assumed to be a homotetramer, only one common model was found, by restraining the

		3	8					13					18					23							
Guinea pig	743	L	A	L	I	G	L	I	I	L	V	I	L	L	L	L	V	I	C	A	I	C	L	CAA77783	
Orangutan	744	M	I	L	S	F	I	I	L	F	I	I	L	L	L	S	T	I	I	C	L	L	C	L	CAA75659
Macaque_all	755	L	V	Y	I	V	P	L	L	L	L	A	L	F	A	G	L	I	L	L	A	S	M	G	CAA56204
Mouse_all	705	L	I	V	L	A	V	I	L	V	L	M	I	L	L	I	I	I	C	I	I	S	A	Y	NP_742123
Bovine	741	I	I	I	P	L	A	A	I	L	C	I	A	L	L	L	I	I	F	C	V	C	T	AAC62752	
Mouse_al	741	V	M	V	L	V	V	P	I	F	L	V	V	L	L	C	C	L	M	L	I	A	Y	L	BAB86768
Rat	743	V	V	V	L	V	V	P	I	F	L	I	V	L	L	C	C	L	M	L	I	A	Y	L	CAA69908
Tamarin_al	744	V	A	V	V	L	V	P	L	F	L	L	I	L	L	C	T	L	L	T	M	S	Y	L	CAA75665
Macaque_al	744	L	V	V	L	V	P	L	F	L	V	L	L	L	L	C	S	L	L	T	I	S	Y	L	CAA56203

Figure 1. Sequences corresponding to ADAM1-TM homologs used in our molecular dynamics simulations. The first column indicates their common name, and the second column is the numbering corresponding to the first residue in the row for each species. The corresponding numbering used here for the synthetic labeled ADAM1-TM (Fig. 2) is shown at the *top*. In mouse and macaque, two copies of ADAM 1 have been found, and they are indicated here as αI and αII. The corresponding NCBI entries are indicated at *right*.

helix tilt to 35°, and only for a right-handed configuration. No other complete sets were found for other tilts or left-handed configurations. The helix tilt for this model was 34° and the rotational orientation ω for residue L14 was 73°. Thus, three models in total were found to be evolutionarily conserved with ω values -77°, 77°, and 178°. Clearly, using site-specific infrared dichroism (Arkin et al. 1997; Torres et al. 2000a) (see below), we can easily discriminate between these models, as the error in the ω determination is only 20°.

Gel electrophoresis

Electrophoresis of the α-helical TM domain shown in Figure 2 is shown in Figure 3 (upper panel). This figure shows that four oligomeric forms of ADAM1-TM were present after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A more precise estimate of the molecular mass corresponding to each band was obtained from the plot of the logarithm of the molecular mass versus the R_f of the markers shown in Figure 3 (lower panel). The expected molecular mass of ADAM1-TM monomer is 3.3 kDa, whereas this graph shows bands consistent with dimeric (6.2 kDa), trimeric (10.8 kDa), pentameric (15.6 kDa), and hexameric (20.0 kDa) homooligomers. Similar homooligomers were observed in the presence of dithiothreitol (DTT), and boiling the sample for 5 min (data not shown), indicating that ADAM1-TM has a high tendency to homooligomerize. Furthermore, these interactions take place without the involvement of disulfide bonds.

Infrared dichroic data

Figure 4 shows the infrared spectra of ADAM1-TM reconstituted in lipid bilayers hydrated with D₂O. The left panel in this figure shows the amide A spectra for the labeled peptides at L14 and L15. The amide I and the band corresponding to the labeled ¹³C=¹⁸O residue (L14 or L15)

is shown in Figure 4 (right panel). It is apparent from the spectra that ADAM1-TM exhibits a highly symmetrical and narrow amide I band, centered at 1655 cm⁻¹, which is consistent with an α-helical peptide (Byler and Susi 1986). No significant intensity around 1640–1630 cm⁻¹ was observed, indicating the absence of β structure (Byler and Susi 1986). Labeling with ¹³C=¹⁸O shifts the carbonyl vibration to a lower frequency, centered at 1591 cm⁻¹. A small band centered at 1618 cm⁻¹ corresponds to remaining ¹³C=¹⁶O that has not exchanged. This does not affect the analysis, as the determination of dichroism of the label is independent from its relative abundance.

As two labels with different ω (L14 and L15) were used, we obtained, as described in the Appendix, two equations for each label; one corresponding to the dichroism of the helix and the other to the dichroism of the label, giving a total of four equations. The dichroic ratios for each sample are listed in Table 1.

Solving the system of equations, the average ω obtained was -50° (±4.3°) and the helix tilt β was 36.4° (±2.1°). The fractional order parameter, f , was always around 0.7. The orientation obtained is only in agreement with the trimeric model with ω of -77° (see above), which is shown in Figure 5.

The ATR cell in our lab cannot be temperature controlled, and the deposited DMPC ($T_m = 23^\circ\text{C}$) bilayers were found to form gel-phase although the FTIR measurements were performed at room temperature. This can be monitored at the lipid symmetric and antisymmetric methylene stretching vibrations which centered at

KK-LALIGLIILVIL**LL**LLLVICAICLGIPA-KK
 KK-LALIGLIILVIL**LL**LLLVICAICLGIPA-KK
 1 5 10 15 20 25 30

Figure 2. Sequences of the synthetic peptide encompassing ADAM1-TM (residues 3–29) used in this study. The residues are numbered from the N- to C-terminal. The isotopically labeled residues are indicated in bold (residues L14 and L15).

Figure 3. (Upper panel) SDS-PAGE electrophoresis of ADAM1-TM. Lane 1, molecular mass markers; lanes 2–4, increasing load of peptide, 5, 10, and 20 μg , respectively. Arrows indicate the bands consistent with dimer, trimer, pentamer, and hexamer forms of the peptide. (Lower panel) Graph of the logarithm of molecular mass (open circles) versus the relative mobility R_f of the molecular mass markers. The filled circles indicate the mobility and the corresponding logarithm of molecular mass of different oligomers of ADAM1-TM.

2849 cm^{-1} and 2917 cm^{-1} , respectively, for lipid in gel phase (data not shown). To obtain the peptide conformational orientation in a system that is close to biological membrane, we also performed the FTIR measurements of peptide reconstituted in POPC ($T_m = -2^\circ\text{C}$) which form liquid crystalline phase at room temperature. The lipid symmetric and antisymmetric methylene stretching vibrations were shifted to 2852 cm^{-1} and 2922 cm^{-1} , respectively (data not shown), and these are consistent with the transitions of the lipid from gel to liquid crystalline phase (Tamm and Tatulian 1997). The dichroic ratios for each sample in POPC are listed in Table 1. With these data, we have obtained an average ω of $-53.4^\circ (\pm 4.0^\circ)$ and the helix tilt β was $38^\circ (\pm 1.7^\circ)$ for ADAM1-TM reconstituted in POPC. Again, this orientation obtained is in good agreement with the trimeric model with ω of -77° .

Discussion

In this work, we have explored the possibility that ADAM 1, which has been proposed to be involved in the fusion event between sperm and egg, and which contains a putative fusion peptide, may form homotrimers, in common with other viral fusion proteins. In particular, we have examined the possibility that these interactions are mediated, at least

in part, by the transmembrane domain. Although various oligomeric forms are observed in SDS (Fig. 3), the rotational orientation for the peptide incorporated in lipid bilayers is consistent only with one model. Indeed, by comparison with the models obtained from molecular simulations of ADAM1-TM, two homotrimers, and one homotetramer, the data is only compatible with one of the homotrimers. Interestingly, the study of the insertion of the fusion peptide of ADAM 1 in lipid bilayers has suggested that the insertion is in a trimeric or dimeric complex, and that ADAM 1 would aggregate for insertion (Wolfe et al. 1999).

Although at present only the heterodimeric form of ADAM 1 and ADAM 2 has been reported, it is possible that the homotrimeric structure form of ADAM 1 could be present during the interaction of sperm and egg. There is evidence, however, that ADAM 1 is not essential in fertilization (Cho et al. 2000). Indeed, ADAM 1 knockout mice were found to be fertile (Kim et al. 2006). It is possible that, being sperm–egg fusion a multistep event, and being so critical in life process, more than one fusion protein may be involved. Indeed, other proteins, such as Izumo on the sperm and CD9 on the egg, have been also identified as fusion proteins (Inoue et al. 2005; Rubinstein et al. 2006).

Alternatively, oligomerization of ADAM 1 may be important to regulate the activities of ADAM 1 in other tissues, as proposed by Wolfsberg and White (1996), because ADAM 1 is not only expressed in testis. It is also expressed at low level in a variety of tissues such as brain, heart, kidney, liver, lung, skeletal muscle, spleen, and ovary (Wolfsberg et al. 1995). The functions and regulation of ADAM 1 in these tissues is unknown. Homooligomerization may transform this membrane protein into a biological active complex as in the case of receptor tyrosine kinases and viral ion channels (Arkin 2002).

The 36° α -helix tilt we have obtained in this study is not common for transmembrane α -helices, although similar tilts have been reported for transmembrane α -helices in other fusion proteins, e.g., synaptobrevin (35°) (Bowen and Brunger 2006) or influenza hemagglutinin (37°) (Tatulian and Tamm 2000). Therefore, it is possible that this high tilt is necessary at some point in the fusion process. Only future experiments will clarify these points.

Materials and methods

Peptide purification

Synthetic peptides, 31 residues long, corresponding to the transmembrane segment of ADAM 1 (residues 743–769, here labeled 3–29) were obtained by standard solid-phase fluorenylmethyloxycarbonyl (Fmoc) chemistry with acetylated N terminus and amidated C terminus, cleaved from the resin with trifluoroacetic acid (TFA) and lyophilized. To improve the

Figure 4. Polarized ATR-FTIR absorbance spectra of ADAM1-TM reconstituted in DMPC bilayers, collected at parallel (solid line) and perpendicular (broken line) polarization. Amide A region of L14 (*upper left*) and L15 (*lower left*). Amide I region of L14 (*upper right*) and L15 (*lower right*). The position of the band of the labeled residue is indicated with an arrow, and displayed as enlarged view shown at the *top right* corner.

solubility of the peptide, the TM was flanked by two lysine residues at each terminus (N and C), as this has been shown not to affect the peptide secondary structure or its oligomerization (Melnik et al. 2001). Two peptides were synthesized (Fig. 2), each containing one $^{13}\text{C}=^{18}\text{O}$ -labeled carbonyl at positions L14 or L15.

The lyophilized peptides were dissolved in a minimum amount of TFA (<10 μL) followed by dilution to 1 mL of 30% isopropanol to a final peptide concentration of ca. 5 mg/mL and immediately injected onto a Zorbax C18-300 Å column (Phenomenex) connected to a high-performance liquid chromatography (HPLC) system (Shimadzu). The solvents used were: solvent A (water/TFA, 99.9:0.1, v/v) and solvent B (isopropanol/water/TFA, 95:5:0.1, v/v). The column was previously equilibrated with a mixture of solvents A and B (6:4, v/v). The peptide was eluted with a linear gradient to a final solvent composition of 75% of solvent B. Pooled fractions were lyophilized and the purity of the samples was checked by electrospray ionization mass spectrometry (ESI-MS), which did not show the presence of adducts (data not shown).

$^{16}\text{O}/^{18}\text{O}$ exchange

The exchange of $^{13}\text{C}=^{16}\text{O}$ to $^{13}\text{C}=^{18}\text{O}$ was performed as described previously (Torres et al. 2000a). Briefly, the two oxygen atoms in the carboxylic group of $^{13}\text{C}=^{16}\text{O}$ -labeled leucine (Cambridge Isotopes Laboratories) were exchanged to ^{18}O by incubating the amino acid at 100°C at acidic pH conditions (pH ~1) with a mixture of H_2^{18}O and dioxane (3:1, v/v) for 1 h. The extent of exchange was monitored using mass spectrometry. The solution was lyophilized and the amino acid was derivatized with FMOc as described (Wellings and Atherton 1997).

Gel electrophoresis

The peptide sample was subjected to SDS-PAGE using 15% tricine gel, in the presence or absence of DTT. SDS sample buffer was added to the lyophilized peptide to a final concentration of 2 $\mu\text{g}/\mu\text{L}$. The sample was mixed with sample buffer for 1 min followed by heating at 95°C for 5 min before loading to the gel. The loading volumes were 5, 10, and 20 μL . The gel was run at constant voltage of 100 V for 1.5 h. The molecular mass markers were obtained from Bio-Rad. The gel was stained with Coomassie blue. The exact molecular mass for a band was determined from the logarithm of the molecular mass versus the relative mobility (R_f) graph.

Table 1. Dichroic ratios measured by SSID using the amide A and the isotopic label for ADAM1-TM incorporated in DMPC and POPC lipid bilayers

Measurements	L14		L15	
	R_{helix}	R_{site}	R_{helix}	R_{site}
DMPC				
a	3.0	5.7	3.0	5.0
b	3.0	5.4	3.0	5.8
c	3.2	4.6	3.4	6.0
POPC				
a	3.33	5.5	2.92	5.4
b	2.8	5.2	3.27	7.7
c	3.25	5.2	2.92	5.3

The information from each labeled residue was obtained from at least three independent measurements.

Figure 5. Evolutionarily conserved model of guinea pig ADAM1-TM homotrimer in agreement with the dichroic data. The corresponding sequence is shown on the *right*, with the residues that are not involved in the interaction indicated.

Infrared spectroscopy

FTIR spectra were recorded on a Nicolet Nexus 560 spectrometer purged with N_2 and equipped with a MCT/A detector cooled with liquid nitrogen. Attenuated total reflection (ATR) spectra were measured with a 25-reflections ATR accessory from Graseby Specac and a wire grid polarizer (0.25 mM, Graseby Specac). Approximately 100 μ L of sample in water with 20:1 lipid/peptide molar ratio were applied onto a trapezoidal (50 mm $\times$ 2 mm $\times$ 20 mm) Ge internal reflection element (IRE). The lipid used here is DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids). A dry, or 2H_2O saturated, N_2 stream flowing through the ATR compartment was used to remove bulk water or to achieve 2H_2O exchange, respectively. After insertion of the plate in the ATR cell, spectra were collected. A total of 200 interferograms collected at a resolution of 4 cm^{-1} were averaged for every sample and processed with one-point zero filling and Happ-Genzel apodisation. The area corresponding to the $^{13}C=^{18}O$ (isotope-labeled) carbonyl stretching vibration was obtained integrating the band at 1590 cm^{-1} . The area of the amide A (N-H stretching, centered at ~ 3300 cm^{-1}) was obtained by peak integration from 3200 cm^{-1} to 3400 cm^{-1} . No difference in band area was observed employing other means of peak size estimation such as peak fitting and Fourier self-deconvolution. The helix dichroism was measured from the amide A when the sample was hydrated in 2H_2O . The dichroic ratio of the band was calculated as the ratio between the integrated absorptions of the spectra collected with parallel and perpendicular polarized light.

Data analysis

The data was analyzed according to the theory of site-specific dichroism, described briefly in the Appendix.

Global search molecular dynamics (GSMD) protocol

The simulations were performed using a Compaq Alpha Cluster SC45, which contains 44 nodes. All calculations were carried

out using the parallel version of the Crystallography and NMR System (CNS Version 0.3), the Parallel Crystallography, and NMR System (PCNS) (Brunger et al. 1998). The global search was carried out in vacuo with united atoms, explicitly describing only polar and aromatic hydrogen atoms as described elsewhere (Adams et al. 1995) using CHI 1.1 (CNS Helical Interactions). For homo-oligomers the interaction between the helices was assumed to be symmetrical.

Trials were carried out starting from either left or right crossing angle configurations. The initial helix tilt, β , was restrained to 5° and the helices were rotated about their long helical axes in 10° increments until the rotation angle reached 350° . Henceforth, the simulation was repeated by increasing the helix tilt in discrete steps of 10° , up to 45° . We must note that the restraint for the helix tilt is not completely strict, i.e., at the end of the simulation a drift of up to $\pm 5^\circ$ from the initial restrained value could be observed in some cases. Three trials were carried out for each starting configuration using different initial random velocities. Clusters were identified for each tilt with a minimum number of similar structures. Any structure belonging to a certain cluster was within 1.5 Å root mean square deviation (RMSD) from any other structure within the same cluster. Finally, the structures belonging to each cluster were averaged and subjected to energy minimization. These final averaged structures were taken as the representatives of the respective clusters. The tilt angle of the models, β , was taken as the average of the angles between each helix axis in the bundle and the bundle axis. The bundle axis, coincident with the normal to the bilayer, was calculated by CHI. The helix axis was calculated as a vector with starting and end points above and below a defined residue, where the points correspond to the geometric mean of the coordinates of the five α carbons N-terminal and the five α carbons C-terminal to the defined residue. The rotational orientation angle ω of a residue is defined by the angle between a vector perpendicular to the helix axis, oriented toward the middle of the peptidic C=O bond of the residue, and a plane that contains both the helical axis and the normal to the bilayer. In this work, to compare the models, a residue was chosen arbitrarily, and the ω angle is always given for residue 14 (corresponding to the first labeled residue). Intersequence comparisons between low-energy clusters were performed by calculating the RMSD between their α -carbon backbone. Fitting was performed using the program ProFit (<http://www.bioinf.org.uk/software/profit>). The energies calculated correspond to the total energy of the system, including both bonded, for example, bond, angle, dihedral, improper, and nonbonded, that is, van der Waals and electrostatic terms (Adams et al. 1995). The interaction energy for the residues was calculated with the function `chi_interaction` implemented in CHI.

Homologous sequences for fertilin α

A total of nine sequences were used for the simulations (Fig. 1). Homologous sequences were obtained using NCBI HomoloGene search (<http://www.ncbi.nlm.nih.gov/>). The assignment of the transmembrane domain for these sequences was based on the hydrophilicity/surface probability plots (Krogh et al. 2001) and the transmembrane predictions from the TMHMM (Transmembrane Hidden Markov Model) server. According to these predictors, the transmembrane region of these sequences spans 23 residues. The alignment of these sequences in the TM domain is shown in Figure 1. The NCBI (National Center for

Biotechnology Information) accession numbers for these sequences are also included in Fig. 1 (right column).

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Appendix

Site specific dichroism is a technique based on the fact that the measured dichroism, R of a particular transition dipole moment is a function of the sample fractional order, f and the spatial orientation of the dipole, which is defined by the parameters: β , the helix-tilt, α , which relates the transition dipole moment to the helix director, and ω , the rotational pitch angle. The rotational pitch angle ω is arbitrarily defined as 0° when the C=O transition dipole moment, the helix director and the Z-axis all reside in the same plane. The following residue is assumed to be 100° away as in the canonical helix. The angle α is known from fiber diffraction studies, and is 39° for transition dipole moment of the peptidic C=O bond and 29° for the N–H bond (Tsuboi 1962).

From each measurement, two different dichroisms are obtained. The first is R_{Helix_i} , the dichroism that corresponds to the $^{12}\text{C=O}$ dipoles, or N–H in the case of amide A, involved in the helical structure. We note that when the dichroic ratio of the helix is obtained from the amide A dichroism, the dichroic ratio should reflect more accurately the tilt of the transmembrane domain. In fact, the amide A band in these conditions, i.e., the sample being exposed to $^2\text{H}_2\text{O}$, originates only from the transmembrane α -helix that has not exchanged. This dichroism arises from residues distributed around the helical axis, (i.e., one every 100° for a standard α -helix). Therefore, this dichroism is independent of ω , and dependent only on β and f_i :

$$R_{Helix_i}(\beta, f_i) = \frac{e_z^2(f_i K_z + \frac{1-f_i}{3}) + e_x^2(f_i K_x + \frac{1-f_i}{3})}{e_y^2(f_i K_y + \frac{1-f_i}{3})}$$

where κ_x , κ_y or κ_z ($\langle\omega\rangle$) are the rotationally averaged integrated absorption coefficients, f_i represents the fractional order of preparation i . The parameter f is 1 if the sample is completely ordered and zero if completely random. Finally, e_x , e_y , and e_z are the electric field components for each axis given by Harrick (1967) according to a thick film approximation. The thickness of the film was calculated as being $>30 \mu\text{m}$, whereas the amplitude of the evanescent wave decays (at $1/e$ of its initial value) after $1 \mu\text{m}$ in a germanium.

The second dichroism, R_{Site_i} , corresponds to the $^{13}\text{C=}^{18}\text{O}$ i label; consequently, it will be dependent on the ω angle for this particular label:

$$R_{Site_i}(\beta, f_i, \omega) = \frac{e_z^2(f_i K_z(\omega) + \frac{1-f_i}{3}) + e_x^2(f_i K_x(\omega) + \frac{1-f_i}{3})}{e_y^2(f_i K_y(\omega) + \frac{1-f_i}{3})}$$

These two equations are not sufficient to obtain β , ω , and f_i (three unknowns); therefore, a second label is inserted with a different ω . For example, if the label is inserted one residue above or below the first label (for a canonical α -helix there are 3.6 residues per turn) the increment in ω is 100° . Thus, two additional equations can be obtained. One is R_{Helix_j} , dependent on β and f_j , and the other is R_{Site_j} , dependent on β , $\omega + 100^\circ$ and f_j . Solving these four equations for each i and j pair, will yield β_{ij} , ω_{ij} , f_i , and f_j , where β_{ij} and ω_{ij} are the results obtained from the combinations of sample i and sample j (Kukol et al. 1999).

The nonlinear equations were solved with Newton's method as implemented in the Find Root function in Mathematica 3.0 (Wolfram Research). The final values of β and ω were obtained by averaging β_{ij} and ω_{ij} , respectively:

$$\beta = \frac{1}{n^2} \sum_{i=1}^n \sum_{j=1}^n \beta_{ij}$$

$$\omega = \frac{1}{n^2} \sum_{i=1}^n \sum_{j=1}^n \omega_{ij}$$

Note that the maximum dichroism R_{max} for C=O groups in an α -helix was obtained from Equation 1, ($\beta = 0$, $f = 1$, $\alpha = 39^\circ$) and is 4.34 for Ge.

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