Pardaxins are a class of ichthyotoxic peptides isolated from fish mucous glands. Pardaxins physically interact with cell membranes by forming pores or voltage-gated ion channels that disrupt cellular functions. Here we report the high-resolution structure of synthetic pardaxin Pa4 in sodium dodecylphosphocholine micelles, as determined by 1H solution NMR spectroscopy. The peptide adopts a bend-helix-bend-helix motif with an angle between the two structure helices of 122 ± 9°, making this structure substantially different from the one previously determined in organic solvents. In addition, paramagnetic solution NMR experiments on Pa4 in micelles reveal that except for the C terminus, the peptide is not solvent-exposed. These results are complemented by solid-state NMR experiments on Pa4 in lipid bilayers. In particular, 13C-15N rotational echo double-resonance experiments in multilamellar vesicles support the helical conformation of the C-terminal segment, whereas 1H NMR experiments show that the peptide induces considerable disorder in both the head-groups and the hydrophobic core of the bilayers. These solid-state NMR studies indicate that the C-terminal helix has a transmembrane orientation in DMPC bilayers, whereas in POPC bilayers, this domain is heterogeneously oriented on the lipid surface and undergoes slow motion on the NMR time scale. These new data help explain how the non-covalent interactions of Pa4 with lipid membranes induce a stable secondary structure and provide an atomic view of the membrane insertion process of Pa4.

Pardaxins belong to a class of small amphipathic peptides that form part of the defense mechanism secreted by sole fish of the genus Pardachirus (1). These polypeptide are postulated to be shark-repelling and toxic to several different organisms (2, 3). The physiology and pharmacology of pardaxins is rather complex; their effects range from interference with ionic transport in both the epithelium and nerve cells to morphological changes in the synaptic vesicles of lipid membranes (4–6). At minimum inhibitory concentrations (3 to 40 μM), pardaxins are able to kill bacteria, whereas at higher concentrations (>50 μM), they lyse red blood cell membranes. In addition, pardaxins can disrupt the ionic transport of the osmoregulatory epithelium and presynaptic activity in mammals by forming voltage-dependent and ion-selective channels (1, 7, 8).

An important characteristic of these membrane active peptides is their selective interaction with specific lipid membranes. Several mechanistic studies carried out with synthetic lipids suggest that pardaxins interact with the lipid surface by aggregating and forming pores, and eventually causing leakage of the cellular content (4). The widely accepted mechanism for pardaxin interactions with these membranes is the so-called “barrel-stave” model. This is a multistep mechanism in which the peptides are thought to a) bind the membrane in an α-helical structure, b) self-aggregate on the membrane surface, c) insert themselves into the hydrocarbon core of the membrane, and d) recruit more monomers, progressively increasing the size of the pore. Helicity, hydrophobic moment, hydrophobicity, charges, and the angle subtended by the hydrophilic/hydrophobic helix surfaces are all crucial structural parameters that modulate both the activity and selectivity of these membrane active peptides (9, 10).

Several biophysical studies show that the known sequences of pardaxins (Fig. 1) contain a single polypeptide chain with a high propensity to aggregate in aqueous solutions (7, 11). It has been predicted that this family of peptides is composed of two α-helices (from residues 2–10 and 13–27, respectively) that are joined by a short hinge flanked by two prolines at positions 7 and 13. The N-terminal segment of the peptide is thought to be inserted into the hydrophobic core of the lipid membranes, whereas the C-terminal helix probably represents the ion channel-lining segment of pardaxin (1, 12).

Several CD studies have also been carried out on pardaxins. The conclusions of these studies are that pardaxins are generally unstructured in aqueous solutions and become highly helical upon the addition of increasing amounts of organic solvent (such as TFE) or synthetic lipids. In 1991, Zagorski and co-workers...
reported the first atomic resolution structure of pardaxin P2 in organic solvent (13). In a mixture of TFE/H₂O (1:1 ratio), pardaxin P2 adopts an L-shaped conformation with proline-13 that promotes the unwinding of the helix, facilitating the formation of a bend. Both the N- and C-terminal residues are unstructured. Although these studies represent a good starting point for the investigation of pardaxin structures, a more rigorous study of the interactions of pardaxin with lipids is necessary to identify pardaxin’s lipid specificity (1).

Solution and solid-state NMR complement each other in the study of small and medium size membrane polypeptides (14). Whereas solution NMR can give atomic resolution information about polypeptides in lipid micelles, solid-state NMR can complement this information by characterizing the interactions of polypeptides with membrane mimicking models that more closely resemble biological membranes. Our previous solid-state NMR investigation showed that the mechanism of membrane disruption by pardaxin P1 is highly dependent on membrane composition. In the present article, we used both solution and solid-state NMR in micelles and lipids, respectively, to gain an atomic view of pardaxin’s (P4a) membrane insertion process.

MATERIALS AND METHODS

Materials—9-Fluorenylmethoxy carbonyl amino acids were purchased from Applied Biosystems (Foster City, CA) and Advanced ChemTech (Louisville, KY). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Isotopically labeled 9-fluorenylmethoxy carbonyl-based solid-phase methods with an ABI 431A peptide synthesizer (Applied Biosystems) as described previously (12). Isotope Laboratories (Andover, MA). All other chemicals were purchased from Aldrich.

Peptide Synthesis—The carboxy-amide of pardaxin (P4a; denoted as P1a in our previous publication) was synthesized using standard 9-fluorenylmethoxycarbonyl-based solid-phase methods with an ABI 431A peptide synthesizer (Applied Biosystems) as described previously (12). The carboxy-amide of pardaxin (P4a) is identical to that of the peptide isolated from Pardachirus marmoratus (12), P-G-F-A-L-I-P-K-I-S-S-P-L-I-F-K-T-L-L-S-A-V-G-S-A-L-S-S-S-G-G-E (15). The peptide was purified by reversed-phase high pressure liquid chromatography using a C18 column. Peptide purity (>97%) was confirmed by high pressure liquid chromatography traces, amino acid analysis, peptide sequencing, and mass spectrometry.

Sample Preparation—P4a peptide labeled with 15N-Leu19 was used in all samples prepared for solution NMR experiments on micelles, 2H NMR experiments on MLVs, and solid-state NMR experiments on mechanically aligned samples. Samples for REDOR experiments used Pa4 labeled with 13C'-Leu18 and 15N-Leu19. All peptide concentrations are given in all samples prepared for solution NMR experiments on micelles,2H stream of nitrogen and then under vacuum overnight to remove any labeled with 13C'-Leu18 and 15N-Leu19. All peptide concentrations are given in mole percent of the peptide isolated from Pardachirus marmoratus (12), P-G-F-A-L-I-P-K-I-S-S-P-L-I-F-K-T-L-L-S-A-V-G-S-A-L-S-S-S-G-G-E (15). The peptide was purified by reversed-phase high pressure liquid chromatography using a C18 column. Peptide purity (>97%) was confirmed by high pressure liquid chromatography traces, amino acid analysis, peptide sequencing, and mass spectrometry.

Solution NMR—All NMR spectra were recorded using a Varian Inova spectrometer operating at 600.48 or 800.24 MHz at a temperature of 303 K and equipped with an inverse detection triple-resonance probe. Resonance assignments were obtained using 2D 1H/1H total correlation spectroscopy (75-ms mixing time) and 1H/1H NOESY (100-, 200-, and 2000-mixing time) experiments (18). Water suppression was achieved using the WATERGATE technique (19). Spectra were collected with 256 and 1024 complex data points in the t₁ and t₂ dimensions, respectively. Total correlation spectra were acquired using a DIPSI (Decoupling In the Presence of Scalar Interactions)–2 pulse sequence (20, 21). The spectral widths were 8 kHz on both the t₁ and t₂ dimensions.

Proton chemical shifts were referenced to internal 3-(trimethylsilyl)propionic acid. NMR spectra were processed using NMRPipe (22) and analyzed using SPARKY (23). 2D spectra were processed with a sine bell window function shifted by 90°. The data were zero-filled to twice their size before Fourier transformation. In the NOESY spectra, obtained with a mixing time of 300 ms, NOE cross-peaks were integrated and used for the structure calculations. The NOE valencies were calibrated using the average NOE volume from resolved aromatic vicinal protons of F2, and from the H7 and H8 cross-peaks of G23. The NOE volumes were classified as strong, medium, and weak, corresponding to distance restraints of 1.9–2.7, 1.9–3.3, and 1.9–5.0 Å, respectively (24).

Solvent accessibility of the side chain amine signals was determined by deuterium exchanger exchange studies. Samples were prepared by dissolving the lyophilized protein sample in d31-DPC into 2H2O and monitoring the disappearance of the NH peaks by 2D NOESY spectra. After a period of >5 h, most of the resonances of the N-terminal portion of the polypeptide disappeared except for residues 5, 6, and 9. The most solvent-protected region was between residues 14 and 26, whose resonances remained after several days.

Solid-state NMR—All spectra from lipid bilayers were acquired using a Varian/Che magnetics 400 MHz spectrometer operating with 1H, 31P, 13C, 2H, and 15N frequencies of 400.14, 161.978, 100.62, 61.62, and 45.55 MHz, respectively. Home-built (double-resonance for 15N and 31P and single-resonance for 1H experiments) flat-coil probes were used for the mechanically aligned samples. Flat coils were made of 2-mm wide flat copper wire with 1-mm spacing between turns and four to six turns per plate with the bilayer normal parallel to the magnetic field. A commercial Varian/Che magnetics double resonance magic angle spinning probe with a 5-mm glass tube was used to acquire the static 1H spectra of unoriented MLVs samples. Spectra were processed using Chemagnetics Spinipsaw software on a Sun Spare workstation. 13P chemical shift spectra of mechanically aligned bilayer samples were obtained using a chemical shift echo sequence, and a periodical mixing time of 100 μs and a 15N decoupling pulse. Typical 1H 1H NOESY spectra were obtained with 256 and 1024 complex data points in the t₁ and t₂ dimensions, respectively. The 1H/1H NOESY spectra were referenced with respect to 85% H₃PO₄ between glass plates (0 ppm). The FID was processed beginning at the top of the echo with 250 Hz (for d₃1-POPC bilayers) or 100 Hz (for oriented d₄-POPC bilayers) exponential broadening, 50 kHz 13C refocusing 180° pulse was placed at the center of the pulse, and 50 kHz 1H decoupling. Typical 1H 90° pulse lengths were 3–5 μs, and the spectral width was 50 kHz. An average of 128 scans was used with a recycle delay of 3 s. The spectra of oriented samples were referenced with respect to 85% H₃PO₄ between glass plates (0 ppm). The FID was processed beginning at the top of the echo with 100 Hz exponential broadening, zero-filled to 2048 points, and Fourier-transformed. Sample thickness was prepared from each experiment were prepared several times independently, and the spectra were compared to the mean.

For 1H acquisition, a quadrupole echo sequence, 90°-r-90°-r-90°-r-90°-t, was used with a radio frequency field strength of 83.3 kHz. 5,000 transients for MLVs and 2,000 for oriented samples were collected with a recycle delay of 2 s. The free induction decay was processed beginning at the top of the echo with 250 Hz (for d₃1-POPC bilayers) or 100 Hz (for oriented d₄-POPC bilayers) exponential broadening, zero-filled to 4096 points, and Fourier-transformed. Sample thicknesses were obtained from each experiment were prepared several times independently, and the spectra were compared to the mean.

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Structure of Pardaxin in Micelles—Pardaxin Pa4 has been reported to aggregate and form tetramers in aqueous solutions (1, 7). Under our experimental conditions, this polypeptide is mostly insoluble in aqueous solutions at pH ~6.5. The peptide solutions become clear only at a lower pH (~4.5), but the quality of the spectra is rather poor, indicating substantial aggregation (data not shown). These findings are in agreement with previous studies by CD spectroscopy, which reported that in H₂O, pardaxin is mostly unstructured, although it adopts a helical conformation upon addition of increasing amounts of TFE (37, 38, 57, 58). We chose DPC micelles for this work because of the affinity of pardaxin for membranes containing phosphatidylcholine (12, 39). In DPC detergent micelles, Pa4 is soluble up ~1.0 mM. However, the polypeptide resonances become undetectably broad at higher concentrations, hampering NMR spectroscopy studies, all our experiments were carried out using Pa4 in a concentration range between 0.5 and 1.0 mM. Under our experimental conditions, the Pa4 peptide samples were stable for several weeks, as determined by 1D proton spectra.

To assign the backbone and side chain resonances, a combination of ¹H/¹H 2D-total correlation spectroscopy and NOESY spectra at different mixing times were used. The high resolution in these spectra allowed us to assign these resonances completely. Two portions of the NOESY spectrum at 100 ms of mixing time are shown in Fig. 2. The complete resonance assignment is given in the supplementary materials. The fingerprint region (H²-NH cross peaks region) of the total correlation spectroscopy has been assigned, with the exception of Gly-1, Phe-2, Pro-7, and Pro-13, which are absent in this spectrum but present in the 2D NOESY experiment. From analysis of the 2D NOESY spectra, we were able to assign a total of 335 NOEs, 81 of which were intraresidue and 254 were inter-residue NOEs. In addition, hydrogen bond constraints were added for residues 14 through 26. Because the secondary structure at the N-terminal portion of the polypeptide is not a regular α-helix, we omitted the hydrogen bond constraints for residues 5, 6, and 9 in the calculations. All NOEs were unambiguously assigned, and pseudo atoms were employed where appropriate using the center of mass approach when stereo-specific assignments could not be made. The 20 lowest energy structures generated gave a backbone RMSD on the average of 0.28 ± 0.10 Å. Conformers were accepted on the basis of the lowest NOE violations using the “accept.inp” routine included in the XPLOR software package. The 20 structures showed no NOE violations greater than 0.5 Å, no bond angle violations greater than 5°, and no bond length violations greater than 0.05 Å. The covalent geometry of the conformers generated was analyzed using PROCHECK_NMR (34).

Peptide Localization in Micelles—For localization of Pa4 relative to the micelle surface, we used three different paramagnetic agents: MnCl₂ (0.1:1 and 0.2:1 [Mn²⁺]/[Pa4]), 5-doxylstearic acid (1:1 and 10:1 [5-doxyl]/[Pa4]), and 16-doxylstearic acid (10:1 and 20:1 [16-doxyl]/[Pa4]) (35). By observing the subsequent line broadening as a result of paramagnetic effects, it was possible to position the peptide relative to the micelle surface. Paramagnetic effects were estimated by the percentage reduction of the backbone HN-Hα resonances using 2D ¹H NOESY experiments at a mixing time of 100 ms (35, 36).

RESULTS

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to a helical secondary structure. The \(d_{\text{HN}}(i,i+1)\) NOEs are present from residues Phe-2 through Ile-6, Lys-8 through Ser-11, and Leu-14 through Ser-27 with a break between Leu-19 and Ser-20.

Although most of the NOEs are localized in the center of the peptide, several long-range NOEs are also present in the N-terminal region of pardaxin. Nonetheless, the presence of only two \(d_{\text{HN}}(i,i+3)\) connectivities involving residues Ile-6 and Ile-9, and Lys-8 and Ser-11, and one \(d_{\text{HN}}(i,i+4)\) connectivity involving Lys-8 and Ser-12, excludes the presence of a well defined \(\alpha\)-helix in the N-terminal region of the peptide. Moreover, we count a dense population of long-range backbone connectivities in the region 14–28: 11 \(d_{\text{HN}}(i,i+3)\) and 11 \(d_{\text{HN}}(i,i+4)\) connectivities. Although the quality of the spectra allowed us to assign both resonances and the NOEs, it was not possible to obtain accurate values for the J-coupling constants using \(^1\)H/\(^1\)H double quantum-filtered or exclusive correlation spectroscopy experiments because of the intrinsic line broadening of peptides and proteins solubilized in detergent micelles (41–43). Therefore, the constraints for the structure calculations were derived from NOEs and hydrogen bond constraints.

Fig. 5 shows the results from our calculations using the simulated annealing protocol built into XPLOR routines. After a first stage of simulated annealing without Lennard-Jones parameters, the van der Waals interactions were introduced to further refine the structures (see “Materials and Methods”). The resulting structures were analyzed, and those with the lowest conformational energy were selected. These structures are superimposed at the \(C\alpha\) atoms of the segments comprising residues 16–30 (\(-0.45\) Å) or residues 2–12 (\(-0.22\) Å). This shows that the proline residue at position 13 may constitute a point of flexibility between two very well defined domains. The first domain comprises a structured turn from residues 2 through 6 and one turn of a helix from residues 7 through 13. On the other hand, the second structural domain contains a turn (14 through 16), and a well defined \(\alpha\)-helix (17 through 30) with a more disordered C terminus. The two domains form an angle of \(-122 \pm 9^\circ\), making this molecule similar to a “crowbar” rather than the “L-shaped” topology reported by Zagorski and co-workers (13). The energetic and geometric parameters for the 20 lowest energy structures are summarized in Table I. The ribbon representation of Pa4 backbone is reported in Fig. 6 showing the amphipathic nature of the C-terminal helix.

**Conformation of the C-terminal Segment in Lipid Bilayers**—Because a lipid bilayer is considered to be a better model for mimicking a cell membrane than a detergent micelle, the backbone conformation of pardaxin and the peptide-induced disorder were measured using solid-state NMR experiments in phospholipid bilayers. Although solving the entire structure of the peptide in lipid bilayers using solid-state NMR techniques is one of the main goals of the project, in this study, we wanted to compare the site-specific conformations of the peptide in micelles and lipid bilayers. To confirm the helicity of the C-terminal amphipathic segment (residues 17 through 30) of pardaxin as determined in DPC micelles, we used solid-state REDOR experiments. REDOR experiments were performed on MLVs containing 3% pardaxin specifically labeled with \(^{13}\)C-Leu-18 and \(^{15}\)N-Leu-19. The REDOR filtered \(^{13}\)C chemical shift spectrum in Fig. 7 consists of a single peak with a frequency value consistent with the helical conformation of this domain, proving that the peptide has a similar conformation in both
micelles and lipid bilayers. This finding ensures that the structure determined from solution NMR experiments in micelles is biologically relevant.

**Topology of Pardaxin in Micelles**—Pardaxin’s topological orientation with respect to the micelle was determined using line broadening of the NMR resonances upon the addition of different paramagnetic agents. The position of pardaxin with respect to the micelle surface was estimated from the percentage reductions of the HN-H\textsubscript{\textsuperscript{1}}H\textsubscript{\textsuperscript{9}} NOESY cross-peaks for the backbone and side chains, respectively (35, 36). Fig. 8, A–C, shows the normalized intensity retention upon the addition of Mn\textsuperscript{2+}, 5-doxyl stearic acid, and 16-doxyl stearic acid, respectively. Upon the addition of Mn\textsuperscript{2+} the resonances of residues Ser-12, Leu-19, Val-22, and Gly-23 are only marginally affected, whereas the resonances of residues Ser-127, Ser-128, and Ser-129 become undetectably broad. On the other hand, the addition of 5-doxyl stearic acid causes a decrease in the resonances located in positions Phe-3, Phe-15, and between Ala-21 and Gly-23, whereas no substantial perturbations of the resonances were observed for residues 10–12 and residues 17 and 19. Upon

<table>
<thead>
<tr>
<th>NOEs</th>
<th>Total</th>
<th>335</th>
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<tr>
<td>Intraresidue</td>
<td></td>
<td>81</td>
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<td>Inter-residue</td>
<td></td>
<td>254</td>
</tr>
<tr>
<td>Hydrogen Bonds</td>
<td></td>
<td>12</td>
</tr>
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**Average Energies (Kcal mol\textsuperscript{-1})**

- $E_{\text{root}}$: 143 ± 13
- $E_{\text{NOE}}$: 48 ± 3
- $E_{\text{bond}}$: 9 ± 1
- $E_{\text{angle}}$: 42 ± 2
- $E_{\text{improper}}$: 5 ± 1
- $E_{\text{VDW}}$: 48 ± 2

**Root-Mean-Square Deviation (Å)**

- Superposition Ca F2-G30: 0.53 ± 0.17
- Superposition Ca K16-G30: 0.45 ± 0.24
- Superposition Ca F2-S12: 0.22 ± 0.18

**Ramachandran analysis**

- Residues in most favored regions: 80.7
- Residues in additional allowed regions: 11.6
- Residues in generously allowed regions: 7.7
- Residues in disallowed regions: 0
The addition of 16-doxyl stearic acid, it is possible to observe a progressive decrease in the signal intensities from residue 23 up to residue 2. Taken all together, the paramagnetic mapping experiments indicate that the peptide does not entirely cross the micelle; rather it is inserted in the micellar core with residues 21–23 close to the detergent headgroups and with residues 24–33 more exposed to the bulk solvent. It is interesting that the resonances of Gly-30, Gly-31, Gln-32, and Glu-33 that belong to the C terminus are not accessible to the Mn$^{2+}$ ions. A molecular map of the changes in cross-peak volumes for the different residues is given in Fig. 8D.

**Fig. 8.** Normalized reduction in signal intensity of NOESY spectra of pardaxin P4 in DPC micelles upon interaction with paramagnetic agents. Reductions in signal intensity of HN-Ho NOESY cross-peaks in the presence of 80 μM Mn$^{2+}$ (A), 8.0 mM 5-doxyl stearic acid (B), and 17.0 mM 16 doxyl stearic acid (C). The signal reductions greater than 50% are mapped onto the contact surface of pardaxin (D). Residues that disappear upon addition of Mn$^{2+}$ are colored red. Residues that are quenched (>50%) upon addition of Mn$^{2+}$ are colored orange. Residues quenched completely by 5-doxylstearic acid are colored blue, whereas residues quenched significantly (>40%) by 5-doxylstearic acid are colored turquoise. Residues quenched by 16-doxylstearic acid are colored pale green. The residues only marginally affected by the paramagnetics are colored gray.

In mechanically aligned lipid bilayers containing pardaxin labeled with $^{15}$N-Leu19 were used to obtain the orientation of the peptide. Our previous studies suggested that the C-terminal helix of the peptide has a transmembrane orientation in DMPC, which agrees well with the results obtained from micelles, and a surface orientation in POPC bilayers (12) However, the signal-to-noise ratio from our previous experiments on POPC bilayers was poor, a complication attributed to the interference of the peptide dynamics with cross-polarization (12). Because we had recently demonstrated that the bilayers could be aligned at low temperatures (17), we conducted further experiments on POPC bilayers at various temperatures to confirm the dynamics of the peptide as well as the results obtained from micelles. As shown in Fig. 9, the $^{15}$N chemical shift spectrum consists of a single broad peak (spanning from approximately 60 to 110 ppm), suggesting that the helical segment is not transmembrane in POPC bilayers but has a heterogeneous surface orientation. Improvement in the signal-to-noise ratio at low temperatures (0 and $-10^\circ$C) confirms that the slow dynamics of the peptide (in the millisecond time scale) interferes with solid-state NMR experiments at higher temperatures, particularly with cross-polarization and decoupling during signal acquisition, explaining the poor signal-to-noise ratio in the previously reported $^{15}$N spectrum of pardaxin in POPC bilayers (12).

**Fig. 9.** $^{15}$N chemical shift spectra of mechanically aligned POPC bilayers containing 2 mol % pardaxin.

$^{2}$H Solid-state NMR Spectroscopy of Lipid Bilayers with Pardaxin—$^{2}$H NMR quadrupolar echo spectra of d$_{31}$-POPC multilamellar vesicles with and without pardaxin were obtained. The spectra of unaligned bilayers (data not shown) were de-Paked to obtain oriented spectra (shown in Fig. 10A) with the bilayer normal parallel to the magnetic field (44). Because the spectral lineshapes in the presence of pardaxin are similar to the pure lipid spectra (see Fig. 10A), the peaks in samples containing pardaxin were assigned in the same manner as for pure lipids (45). $^{31}$P experiments were also performed on these samples to confirm that the lipids were in a fluid lamellar phase both before and after the $^{2}$H NMR spectra were obtained (spectra not shown). The quadrupolar splitting is reduced in the presence of pardaxin at all positions along the acyl chain and decreases with increasing peptide concentration. Deuteron order parameters are plotted in Fig. 10B and were calculated from the de-Paked spectra as explained in the literature (25, 45). It is clear that the presence of the peptide increases disorder along the acyl chain and the degree of disorder is dependent on the concentration of the peptide. To measure the
peptide-induced disorder or structural changes in the head group region of lipids, solid-state NMR experiments were performed on mechanically aligned \( \text{d}_4 \)-DMPC bilayers. The \( ^{31} \text{P} \) and \( ^{2} \text{H} \) spectra of \( \text{d}_4 \)-DMPC bilayers with and without peptide given in Fig. 11 reveal a number of interesting features. The presence of pardaxin decreases the \( ^{31} \text{P} \) CSA span, as observed from MLVs (data not shown) as well as from the residual unoriented component of the spectra given in Fig. 11A. The presence of the peptide also decreases \( ^{2} \text{H} \) quadrupole splitting at the \( \text{C}9 \) site, whereas it increases \( ^{2} \text{H} \) quadrupole splitting at the \( \text{C}_\beta \) site. The changes in the \( ^{31} \text{P} \) CSA span and \( ^{2} \text{H} \) quadrupolar splittings will be discussed below.

**DISCUSSION**

**Topology of Pardaxin**—Several different studies point to a “barrel-stave” mechanism of action for pardaxin in lipid membranes. The N-terminal portion of this peptide is thought to be involved in both the insertion of the peptide in the lipid bilayers and in the aggregation process (46, 47). On the other hand, the C-terminal helix, which is markedly amphipathic, is involved in the formation of ion-channels (46, 47). Our previous solid-state NMR studies in mechanically aligned DMPC bilayers suggested that the C-terminal amphipathic helix is transmembrane, which corroborates its role in the formation of ion-channels (12). This observation is in complete agreement with the solution NMR results in detergent micelles as well as consistent with the barrel-stave mechanism of membrane-disruption by pardaxin. On the other hand, the \( ^{15} \text{N} \) solid-state NMR spectrum of pardaxin in POPC bilayers indicates that pardaxin is oriented topologically with the amphipathic C-terminal-helix approximately perpendicular to the bilayer normal (12). It also shows that the peptide undergoes motion on a timescale that interferes with the transfer of magnetization from protons to \( ^{15} \text{N} \), and/or with proton decoupling during signal acquisition in a cross-polarization experiment.

The increase in signal-to-noise ratio at low temperatures and the broad line width of the spectral line obtained from aligned samples (Fig. 9) in the present study suggest that the C-terminal helix has heterogeneous orientations on the surface of the bilayer. Because the hydrophobic thickness of the DMPC and POPC bilayers differs by only 3 Å, this discrepancy cannot be explained by a hydrophobic mismatch alone (48). In addition, both differential scanning calorimetry and \( ^{31} \text{P} \) spectroscopy

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**FIG. 10.** A, dePaked \( ^{2} \text{H} \) NMR spectra of POPC MLVs containing 0, 3, and 5 mol % pardaxin. B, order parameters (\( S_{\text{CD}} \)) measured from spectra given in A.

**FIG. 11.** \( ^{31} \text{P} \) (A) and \( ^{2} \text{H} \) (B) NMR spectra of mechanically aligned \( \text{d}_4 \)-DMPC bilayers containing 0, 3, and 5 mol % pardaxin. Signals marked with asterisks were caused by unoriented lipids in the sample.
shown that pardaxin behaves rather complexly in the presence of different lipids. For instance, the effects of pardaxin on POPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine bilayers are quite different (12), indicating that the difference in the acyl chains between POPC and DMPC is not the only factor that can affect peptide activity. These observations are further supported by $^3$H NMR data in $d_2$-POPC (Fig. 10) suggesting that pardaxin increases disorder in the acyl chain of POPC bilayers in a concentration-dependent manner, which is in good agreement with similar trends observed for other surface-oriented peptides (25). At the same time, under the concentrations used in this study, the peptide does not alter the lamellar phase structure of lipid bilayers, ruling out the detergent-type micellization mechanism of membrane-disruption. The topology and physicochemical properties elucidated here can be correlated with the reported fusogenic property of pardaxin.

Does Pardaxin Induce Changes in the Conformation and Dynamics of the Lipid Headgroup?— $^2$H and $^{31}$P NMR experiments were performed on DMPC bilayers with and without pardaxin to measure the peptide-induced changes in the headgroup region of lipid bilayers. The addition of pardaxin to $d_2$-DMPC bilayers decreases quadrupole coupling at the C$_\alpha$ site, whereas it increases at the C$_\beta$ site (Fig. 11B). These changes can be interpreted in terms of modulation of headgroup dynamics and/or conformation. Because any changes caused by the dynamics of the lipid headgroup (alteration of disorder or order or angular fluctuations) would have the same effect on $\alpha$ and $\beta$ segments, they would cause the quadrupole couplings to change in the same direction for both the carbon sites. The observed counter-directional changes in the quadrupole couplings cannot therefore be explained by the changes in the dynamics of the headgroups. On the other hand, these results can be interpreted in terms of a conformational change in the phosphocholine headgroup. NMR studies have shown that the “P-N” dipole of the phosphocholine headgroups that is oriented almost parallel to the plane of the bilayer surface can be altered in the presence of electric charges. For example, the addition of a cationic amphiphile moves the N$^+$ end of the dipole toward the water phase, whereas an anionic amphiphile has the opposite effect (49). Because the net charge of pardaxin at neutral pH is +2, it could repel the N$^+$ end of the “P-N” dipole vector that would move the dipole toward the water phase of the bilayer, thus changing the phosphocholine conformation. This interpretation is in good agreement with the results reported for the effects of cations on the conformation of the lipid headgroup (49).

According to previous studies (49), a conformational change in the lipid head group may increase the $^{31}$P CSA span; however, the $^{31}$P CSA spans measured from DMPC and POPC bilayers are reduced in the presence of the peptide (Fig. 11A). This observation could be the result of other factors that alter the CSA span (50, 59). In addition, it is unlikely that the peptide is increasing the dynamics at the $^{31}$P site without affecting the C$_{\alpha\beta\delta}$ sites. These results suggest that choline headgroups do not prevent membrane disruption by pardaxin, which correlates well with pardaxin’s toxicity toward eukaryotic cells whose membranes contain zwitterionic lipids.

High-resolution Structure of Pardaxin—The solution NMR investigation of pardaxin in detergent micelles is complementary to solid-state NMR results obtained from lipid bilayers. In particular, the quality of the NMR spectra allowed us to obtain a high-resolution structure for pardaxin in membrane-mimicking environments. The large number of NOE constraints allowed us to define the secondary structure elements and to elucidate the relative orientation of the N- and C- helical domains with greater accuracy. The previously obtained structure of pardaxin was solved using the helix-inducing TFE membrane-mimicking environment. Although this study provided some insights into the structure, it has been reported that the structure-inducing driving forces of isotropic environments (such as organic solvent mixtures) are substantially different from those of non-isotropic phases (such as micelles or vesicles) so that membrane active polypeptides can have different secondary structure elements in different environments. This has been the case for pardaxin, which displays either an $\alpha$-helical or $\beta$-strand arrangement of the backbone depending on the membrane-mimicking system used. Therefore, it is not surprising that the structure presented here differs from the one proposed by Zagorski and co-workers (13).

Although the overall shape of the molecule is substantially similar (bend-helix-bend-helix), there are many differences in each of the secondary structure domains. First, Zagorski and co-workers identified five different structural domains (1–5, 6–11, 12–13, 14–26, 27–33), with two unstructured regions (12–6 and 27–33). In DPC micelles, the N-terminal region of the peptide is highly structured. The two aromatic rings display several NOEs, making this region a well-defined bend. Because the P7A mutant of pardaxin shows enhanced helicity and consequently more pronounced hemolytic activity, the structure of this region might play an important role in the specificity of pardaxin for bacterial over mammalian cells (9, 37). Second, the structure in TFE is L-shaped, with proline 13 causing the helix to unwind. This in turn results in a bend of $-90^\circ$ between the two helical domains. In DPC micelles, the proline plays a similar role except that the angle between these two domains is $122 \pm 9^\circ$. This larger angle may better explain the ability of pardaxin to insert itself in lipid membranes, forming a “funnel” like oligomer in the membrane bilayers. The considerable differences between the two structures in TFE and DPC can be attributed to the effects of alcohols on polypeptide structures in which alcohol weakens non-local hydrophobic interactions, favoring local polar interactions such as hydrogen bonding (9). The disruptive effects of organic solvent mixtures such as alcohol/water have also been demonstrated with larger polypeptides such as phospholamban (42).

Membrane-lytic Activity of Pardaxin—Unlike that of most of the other membrane active antimicrobial peptides, such as LL-37, magainin, MSI-78, or cercepin, the secondary structure of pardaxin is rather complex. Although the above-mentioned polypeptides comprise mainly a single $\alpha$-helix, pardaxin’s secondary structure is a composite bend-helix-bend-helix, with an overall topology similar to that of M2 protein from influenza A virus. A model structure of this protein was recently proposed (51). The structure of the pore-forming monomer was proposed to have two distinct regions: one hydrophobic region, inserted in the hydrocarbon region of the membrane with a tilt angle of $25^\circ$ with respect to the bilayer normal, and the second an amphipathic helix lying on the surface of the bilayer with an angle of $80^\circ$. The angle between the two domains is about $125^\circ$, which is near the topological arrangement of pardaxin, whose angle between the two helical domains is $122 \pm 9^\circ$. These topological similarities suggest that a comparable mechanism might be taking place for the oligomerization of both M2 protein from influenza A virus and pardaxin, with the structure in DPC micelles supporting a mechanism of aggregation consistent with the proposed “barrel-stave” model (47, 52): the N-terminal domain seems to be involved in the insertion in the lipid bilayer, whereas the C-terminal amphipathic helix constitutes the putative ion-channel lining segment.

Although pardaxin has been thought to be a “melittin-like"
peptide even though its toxicity is considerably lower than that of melittin, our study clearly suggests that the structures of pardaxin and melittin (53, 54) differ significantly. Both these peptides have two structural domains, and the angle between the domains is similar. Although this similarity may explain their cytotoxicity, the differences in their structural details could be useful in understanding the differences in their activities. In addition, the mechanisms of membrane-disruption by these peptides are considerably different. Pardaxin induces a negative curvature on lipid bilayers (12), whereas melittin induces a positive curvature strain (55). Melittin lyases membranes into micelles (56), but pardaxin's membrane-disruptive mechanism is dependent on membrane composition. In addition, pardaxin's ability to disrupt lipid bilayers is substantially reduced by the addition of cholesterol, showing that this peptide has a preference for bacterial over mammalian cells, even though its selectivity is poor compared with that of other antimicrobial peptides. This explains why pardaxin, unlike melittin, is selective toward bacterial membranes at low concentrations and toxic to eukaryotic cells at higher concentrations.

CONCLUSIONS

We have determined the high-resolution structure and topology of pardaxin P4 in DPC micelles. Although the overall secondary structure resembles the one proposed by Zagorski et al. (13), there are many differences in the length and nature of each secondary structure domain. The structure presented here shows a mostly structured N terminus, with the residues in a tight bend conformation, and the N-terminal helix nucleated by a proline in position 7 contains only one turn of a helix, which is interrupted by a second proline in position 13. The amphipathic C-terminal helix spans residues 14–30, forming a 122 ± 9° angle with the N-terminal helix. Moreover, we found that the N-terminal domain is solvent-protected, whereas the C-terminal residues 30–33 are solvent-exposed. Residues 21–23 lie beneath the polar heads of the detergent micelles.

The backbone conformation of the C-terminal segment and its topology determined from lipid bilayers using solid-state NMR methods are in good agreement with the results obtained from micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure. 31P and 2H Solid-state NMR data from lipid micelles, further confirming the biological relevance of our pardaxin structure.
Structure and Orientation of Pardaxin Determined by NMR Experiments in Model Membranes

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