Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5872–5876, June 1996
Biophysics

Conformational trapping in a membrane environment: A regulatory mechanism for protein activity?

(molecular memory/^{15}N solid-state NMR/orientational constraints/membrane proteins/membrane protein stability)

S. ARUMUGAM*, S. PASCAL†, C. L. NORTH‡, W. HU§, K.-C. LEE¶, M. COTTEN, R. R. KETCHEM, F. XU, M. BRENNEMAN, F. KOVACS, F. TIAN, A. WANG, S. HUO, AND T. A. CROSS‖

Center for Interdisciplinary Magnetic Resonance at the National High Magnetic Field Laboratory, Institute of Molecular Biophysics, and Department of Chemistry, Florida State University, Tallahassee, FL 32306

Communicated by Michael Kasha, Florida State University, Tallahassee, FL, January 29, 1996 (received for review November 30, 1995)

ABSTRACT **Functional regulation of proteins is central to living organisms. Here it is shown that a nonfunctional conformational state of a polypeptide can be kinetically trapped in a lipid bilayer environment. This state is a metastable structure that is stable for weeks just above the phase transition temperature of the lipid. When the samples are incubated for several days at 68°C, 50% of the trapped conformation converts to the minimum-energy functional state. This result suggests the possibility that another mechanism for functional regulation of protein activity may be available for membrane proteins: that cells may insert proteins into membranes in inactive states pending the biological demand for protein function.**

The aqueous environment for water-soluble proteins promotes conformational rearrangements by mediating hydrogen bond exchange and by inducing dynamics at the solvent-exposed surface (1, 2). Consequently, even minimum-energy conformational states for water-soluble proteins are marginally stable (3, 4). The lipid environment is very different (5). It does not mediate hydrogen bond exchange or promote dynamics; instead, conformational states are stabilized as demonstrated here. Although evidence for kinetically trapped metastable conformational states of certain water-soluble proteins has been growing (6), here the conformation and conversion of a metastable state to a thermodynamically favored state is described and demonstrated for a bilayer-bound polypeptide.

The polypeptide gramicidin A is particularly appropriate for these studies of conformational stability, because it has a large surface to volume ratio. Its molecular structure and dynamics are highly sensitive to its environment. In lipid bilayers, gramicidin typically forms a single-stranded helical dimer (see Fig. 1, blue), whereas in organic solvents, it forms a variety of stable double-helical conformations (see Fig. 1, red) that vary in handedness and symmetry (i.e., parallel vs. antiparallel) (7). These conformers interconvert slowly enough in ethanol that four different conformations are well resolved by solution NMR spectroscopy (8). They convert much more slowly in dioxane or tetrahydrofuran, because the solvent cannot efficiently promote hydrogen bond exchange, and consequently, the conformers are trapped in a metastable state (7, 9, 10). In fact, they can be separated by TLC or normal-phase HPLC, and solution NMR spectroscopy can be performed on the individual conformers (10). The equilibrium between these conformational states varies, depending primarily on the dielectric constant of the solvent (unpublished results). Furthermore, when lipid bilayers containing gramicidin were prepared by first cosolubilizing peptide and lipid in various organic solvents followed by solvent removal and hydration, a solvent history dependence was observed (9, 11, 12). In other words, the use of certain organic cosolubilizing solvents leads readily to the channel state (characterized by ^{15}N and ^{23}Na NMR as well as CD spectroscopy and HPLC), whereas the use of other solvents results in the need for extensive incubation before showing features typical of the channel state. Apparently, conformations of gramicidin that are metastable can be formed in a lipid bilayer environment.

Solid-state NMR is well suited for characterizing proteins in anisotropic environments, and moreover, it can take advantage of uniaxially aligned samples (13–18). The gramicidin channel structure has been solved with solid-state NMR-derived orientational constraints from samples in fully hydrated lipid bilayers (13). Through isotope labeling of individual specific amino acid sites, orientation-dependent nuclear spin interactions have been monitored in aligned samples (see Fig. 1). Because of the many precise constraints observed for the gramicidin channel, its structure is one of the highest resolution characterizations of a membrane-bound polypeptide or protein structure.

MATERIALS AND METHODS

Isotopically labeled amino acids were purchased from Cambridge Isotope Laboratories (Cambridge, MA). Dimyristoyl phosphatidylcholine was purchased from Sigma. Both amino acids and lipids were used without further purification. Gramicidin A was synthesized using fluorenylmethoxycarbonyl blocking chemistry and a peptide synthesizer (Applied Biosystems; model 430A) (19). Peptides that were synthesized in <98% purity were purified by semipreparative HPLC as described (19). Oriented samples were prepared using a gramicidin/lipid molar ratio of 1:8. The peptide and lipid were cosolubilized in an organic solvent, and the sample was dried on thin glass plates. Approximately 20 plates were stacked in a segment of square glass tubing that was sealed following the addition of ≈50% (wt/wt) water. The sample was then incubated for 14 days at 45°C to complete the hydration of the bilayers without resorting to agitation.

^{15}N solid-state NMR spectra were obtained on a heavily modified spectrometer (Bruker, Billerica, MA; model WP200 SY) with a DOTY Scientific (Columbia, SC) solid-state accessory. The ^{15}N frequency was 20.3 MHz, and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*Present address: Department of Biochemistry, University of Missouri, Columbia, MO 65211.
†Present address: Department of Medical Genetics, University of Toronto, ON Canada M5S-IA8.
‡Present address: Department of Chemistry, University of Virginia, Charlottesville, VA 22901.
§Present address: Biophysics Research Division, University of Michigan, 930 N. University Avenue, Ann Arbor, MI 48109-1055.
¶Present address: Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.
‖To whom reprint requests should be addressed.

typical parameters for cross-polarization were 6-μs 90° pulse widths, 1-ms mixing time, a 7-s recycle delay, and an 8-μs dwell time. High power ^{1}H decoupling was employed during data acquisition. Typically, 10,000 acquisitions were obtained for each spectrum.

RESULTS AND DISCUSSION

Fig. 1 shows the origin of the solid-state NMR-derived orientational constraints. The gramicidin structures are all based on β-sheet type hydrogen bonding, and therefore, the repeating

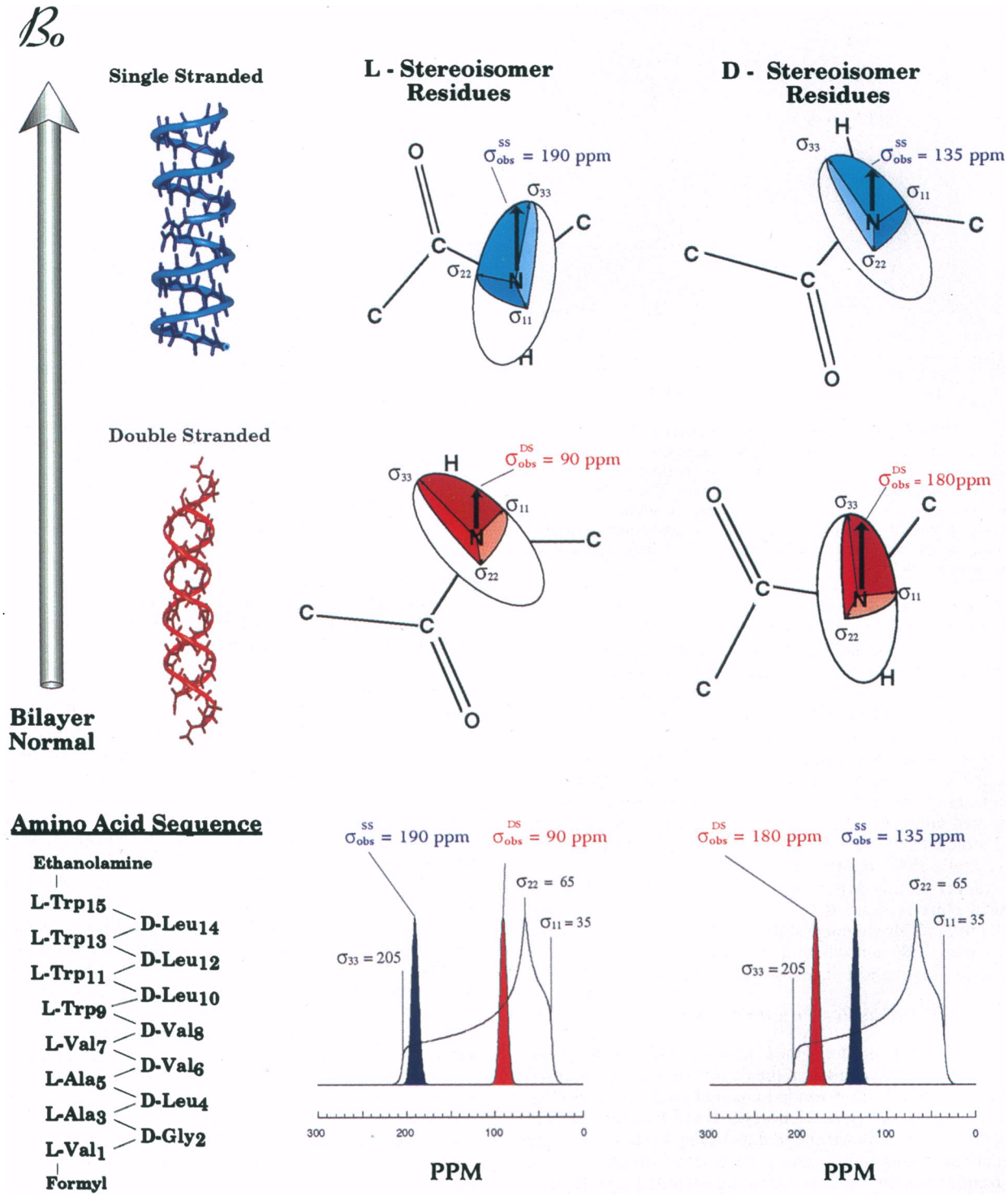

FIG. 1. Gramicidin forms a variety of dimeric structures based on β-sheet type hydrogen bonding. Because of its alternating amino acid stereochemistry, the side chains are on one side of the sheet structure, thereby forcing it into a helix. The structural repeat, typical of a β-sheet, is a dipeptide with the carbonyl groups alternating between parallel and antiparallel to the channel axis. In an anisotropic environment the chemical shift of the NMR spectrum is orientation-dependent with respect to B_o. This dependence is described by the chemical shift tensor, which is fixed in the peptide plane and illustrated here by ellipsoids with axes of σ_{11}, σ_{22}, and σ_{33}. For the single-stranded conformation (blue) in oriented bilayers where the helical axis and bilayer normal are aligned parallel to B_o, the observed $^{15}N_\alpha$ chemical shifts are 190 ± 10 ppm for the L residues and 135 ± 10 ppm for the D residues. For the double-stranded helix (red) a similar repeating pattern is predicted based on a solution NMR structure (20) and a crystal structure (21). For this helix, the $^{15}N_\alpha$ chemical shifts are predicted to be ≈90 ppm for the L sites and ≈180 ppm for the D site frequencies. Simulated spectra of unoriented samples show the full range of orientation-dependent chemical shifts with the spectral discontinuities defining the tensor axes.

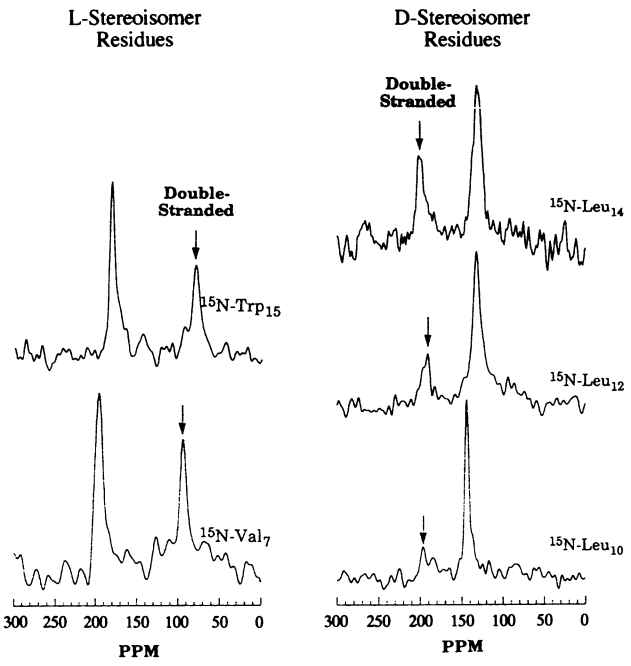

FIG. 2. ^{15}N spectra of uniformly aligned dimyristoyl phosphotidylcholine bilayers containing single-site ^{15}N-labeled gramicidin A. These samples were prepared by cosolubilization of the lipid and peptide in a mixed solvent of methanol/water (98:2, vol/vol). The dominant peak in each spectrum is from the channel conformation. The minor resonances marked by arrows are due to a new conformation consistent with the chemical shift predictions in Fig. 1 for a left-handed double-stranded helix.

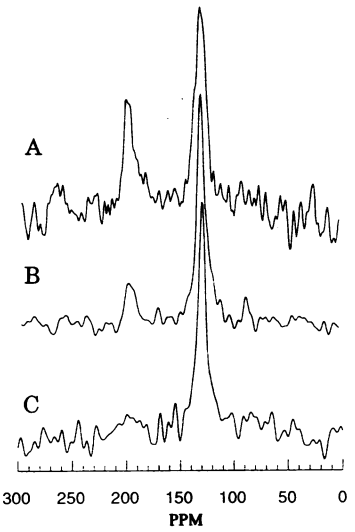

FIG. 3. ^{15}N NMR spectra of ^{15}N Leu-14-labeled gramicidin A in oriented lipid bilayers. (*A*) Sample initially cosolubilized in methanol/water (98:2, vol/vol) where the polypeptide is known to exist as a complex mixture of parallel and antiparallel double-helical conformations. In lipid bilayers, as shown in Fig. 2, two conformations are observed. (*B*) The sample in *A* after it has been incubated at 68°C for 3 days. (*C*) Sample initially cosolubilized in benzene/ethanol (95:5, vol/vol) where the polypeptide is known (20) to exist as a single dimeric conformation, an antiparallel double-stranded helix with a left-handed helical sense. In lipid bilayers, a single conformer is observed that has previously been shown to be the right-handed single-stranded helix of the channel state.

structural unit is a dipeptide consisting of one L residue and one D residue. The orientation of the amide ^{15}N D and L sites alternate in a regular pattern about a helical axis generating an alternating pattern of ^{15}N chemical shifts observed from oriented samples. The observed chemical shifts are specified by the orientation of the ^{15}N chemical shift tensors, which are shown in the figure as ellipsoids orientationally fixed in the molecular frame. The orthogonal tensor elements that describe the axes of each ellipsoid have magnitudes defined by frequencies shown in the simulated powder pattern spectra (of unoriented samples) at the bottom of the figure. Consequently, as the molecular frame orientation is changed with respect to the magnetic field, B_o, the chemical shift tensor orientation with respect to this axis is also changed, and the observed chemical shift is defined by the component of the tensor parallel to B_o. This chemical shift can also be described by the sum of tensor element components, where θ_{ii} represents the orientation of the tensor elements with respect to B_o, as follows:

$$\sigma_{obs} = \sigma_{11}\cos^2\theta_{11} + \sigma_{22}\cos^2\theta_{22} + \sigma_{33}\cos^2\theta_{33}.$$

The orientation of the amide sites depends on both the handedness and helical pitch of the conformers. Consequently, the right-handed single-stranded channel conformation (Fig. 1, blue) gives rise to very different observed frequencies than the left-handed double-stranded dimer (Fig. 1, red). A change in handedness causes the L and D residues to switch chemical shift frequencies; in other words, a right-handed double helix would have L residue frequencies of 180 ppm and D residue frequencies of 90 ppm. The doubling of the helical pitch between single- and double-stranded dimers results in an approximate doubling of the frequency separation between L and D residues.

Fig. 2 shows spectra obtained from a variety of single-site isotopically labeled gramicidin samples in lipid bilayers prepared with a methanol/water cosolubilizing solvent. In each spectrum, the dominant resonance is consistent with the published chemical shift of the channel conformation for that particular site (13). The spectra of D and L residues have been separated as in Fig. 1. The channel conformation has L-site resonances near 190 ppm and D-site resonances near 135 ppm. Based on this alternating pattern of chemical shifts, a β-sheet structure was confirmed, and the helix sense was unambiguously determined to be right-handed (22). The other resonance in each spectrum in Fig. 2 (arrow) is associated with a different conformation, and because the resonances are narrow for this new conformation, it is also a structure that is well aligned with respect to the lipid bilayer. The alternating pattern of chemical shifts suggests both a β-sheet structure and a helix of opposite sense to that of the channel state. Furthermore, the much greater separation of L- and D-site frequencies dictate a helical pitch typical of a double-stranded structure. The resonance frequencies for this new conformational state are consistent with those predicted from either a parallel or antiparallel double-stranded conformation with a left-handed helical sense.

When ^{15}N Leu-14-labeled gramicidin samples are cosolubilized in benzene/ethanol, a single conformation, the channel state, results as shown by a single sharp resonance at 131 ppm in the ^{15}N NMR spectrum (Fig. 3*C*). When the cosolubilization solvent is methanol/water, as in Fig. 2, two resonances are observed for each labeled site (Fig. 3 *A* and *B*), and when this sample is incubated at 68°C for 72 h, the intensity in the new resonance diminishes by more than 50% (Fig. 3*B*). Therefore, this resonance and others like it in Fig. 2 represent a kinetically trapped metastable conformation that, upon extensive incubation at elevated temperatures, can be converted to the minimum-energy conformation in the bilayer environment (the channel state).

It is well known that the left-handed antiparallel double helix (Fig. 4) that dominates (>95%) in benzene/ethanol solvent mixtures is readily converted in hydrated lipid bilayers (12) to the channel state, a single-stranded conformation with a right-handed helical sense. The proposed mechanism for this conformational rearrangement includes an untwisting of the double helix (25–29), thereby minimally exposing the amide

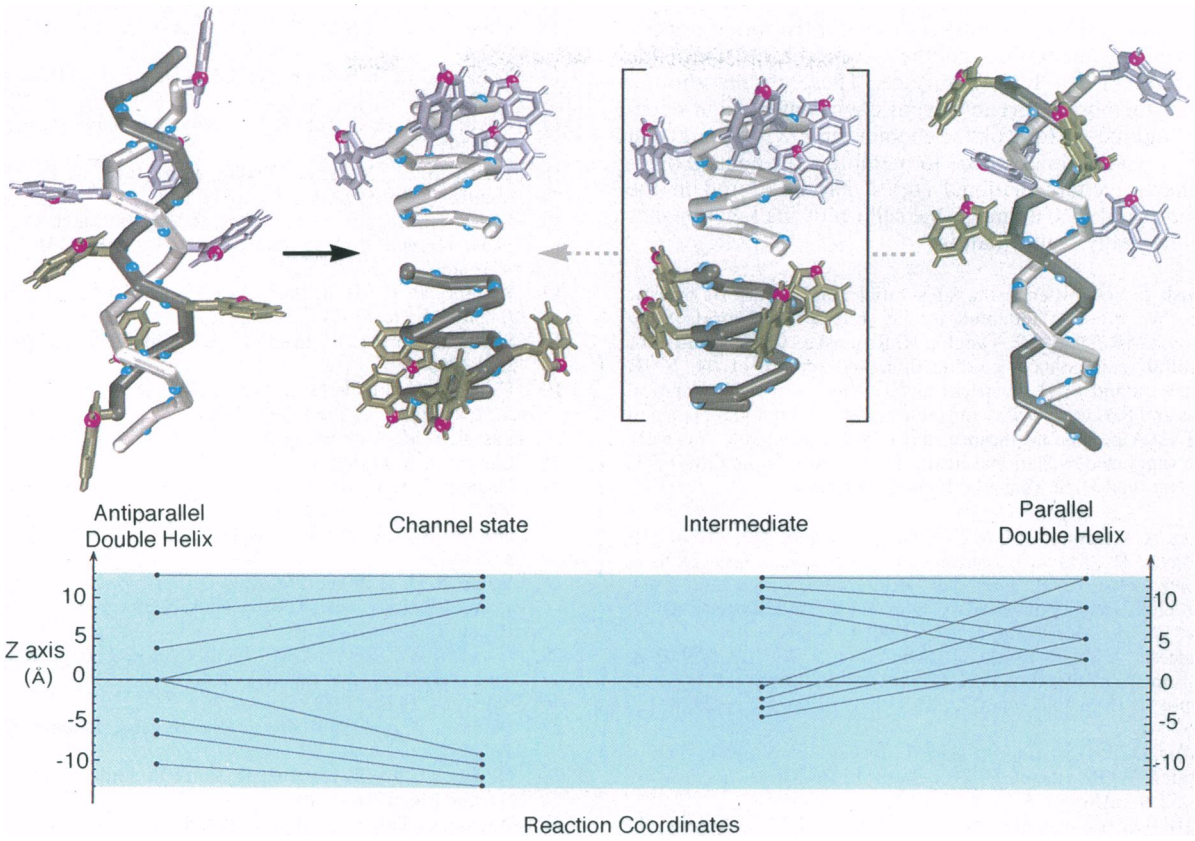

FIG. 4. Conformational rearrangements of gramicidin dimers within a lipid bilayer. The backbone is drawn to emphasize the β-sheet nature of these structures, The C^{α}–C^{α} vector alternates in orientation to the bilayer normal, and the amide nitrogens shown in cyan alternate on either side of the tube representing the backbone. It is the spectroscopy of these sites that has provided the data for this paper. The antiparallel double-helix structure is a representation of the solution NMR-derived structure (20). The channel state is a representation of the high-resolution solid-state NMR-derived structure in lipid bilayers (13). The backbone conformation of the parallel double helix is based on results from Bystrov and coworkers (8), but the side-chain orientations are model-based without experimental verification. The intermediate is shown only to illustrate the burying of indoles deep within the bilayer; the structure is hypothetical. The antiparallel double helix converts readily to the channel state in lipid bilayers, whereas the parallel double helix is trapped in a metastable state. The driving force for such conformational rearrangements lies in the movement of the indole nitrogens (shown in magenta in the structures) toward the bilayer surface and away from the low dielectric environment of the bilayer center as illustrated at the bottom of the figure, where the colored area approximates the hydrophobic width of the liquid crystalline state of the dimyristoyl phosphatidylcholine bilayers (23, 24). With considerable sample heating, it is possible to convert the kinetically trapped state to the channel conformation, hence the dotted arrow connecting the parallel double helix with the channel state. A high activation energy barrier, due to the flip-flop of four indoles across the bilayer, for the conversion of the parallel double-stranded structure to the single-stranded channel state is the reason why the parallel double-stranded structure is trapped in a metastable state.

carbonyls to the low dielectric lipid environment. The change in helix sense is accomplished simultaneously by a 180° flip of each peptide plane (about an axis approximated by the C^{α}–C^{α} vector) as it is freed from hydrogen bonding to the intertwined strand (29). This results in switching the side chains from one side of the β-sheet to the other, forcing the sheet to curl with the opposite helical sense. The amino-terminus to amino-terminus junction of the channel state is directly achieved by this mechanism initiated with an antiparallel double helix. The driving force for this conformational rearrangement results from the disposition of the indole rings within the lipid environment. The channel state has each of the indole NH groups oriented toward the hydrophilic bilayer surface, where the hydrogen can exchange with water in the hydrophilic–hydrophobic interface (14). As illustrated at the bottom of Fig. 4, the antiparallel structure in rearranging to the channel state moves the indole nitrogens to the bilayer surface when the structure untwists in the direction of forming the amino-terminus to amino-terminus dimer.

In methanol, both antiparallel and parallel double helices exist (7, 8). When bilayers of gramicidin are prepared using methanol as a cosolubilizing solvent, two conformations are observed, one of which is metastable and double-stranded with a left-handed helical sense. For a parallel conformer to convert to the channel state, an additional rearrangement step is needed (as shown on the right side of Fig. 4), because the untwisting of such a structure results in an amino-terminus to carboxy-terminus junction and the burial of four indoles deep within the bilayer. These significant energetic costs are akin to the energetic cost of flipping a cholesterol molecule between bilayer leaflets, the rate of which is known to be very slow (30, 31). Consequently, it is judged here that the observed metastable structure is a parallel double-stranded dimer that has all of its indole rings in one bilayer leaflet.

The trapping of a non-minimum-energy conformation is made possible by secluding the polypeptide from the catalytic effect of a protic solvent** and by the low dielectric constant of the fatty acyl chain environment. Such a low dielectric stabilizes electrostatic interactions, including hydrogen bonds. Furthermore, the heterogeneity in this environment arising from the dielectric constant gradient (between the bilayer

**We refer to protic solvents as "catalysts" because these solvents have the ability to promote hydrogen bond exchange that can lead to conformational rearrangements. The solvent is not consumed in the process and the potential energy barrier for conformational rearrangement is lowered, hence the justification for using the term catalyst.

surface and interior) generates a driving force for conformational rearrangement through the need to redistribute the indole rings at the bilayer surface. These results show a molecular memory between solvent environments and documents a unique feature of the membrane environment that may be of general importance to membrane proteins. Potentially, inactive conformational states may be stored in the membrane until such a time as the cell needs the functional or minimum-energy conformation.

We wish to acknowledge the substantial graphics efforts by Eric Fontano. We are also indebted to the staff of the Florida State University NMR Facility, J. Vaughn, R. Rosanske, and T. Gedris, for their skillful maintenance, modification, and service of the NMR spectrometers and to H. Henricks and U. Goli in the Bioanalytical Synthesis and Services Facility for their expertise and maintenance of the ABI 430A peptide synthesizer and HPLC equipment. This work has been supported by National Institutes of Health Grant GM-49092 and the National High Magnetic Field Laboratory.

1. Zaks, A. & Klibanov, A. M. (1988) *J. Biol. Chem.* **263,** 8017–8021.
2. Affleck, R., Xu, Z.-F., Suzawa, V., Focht, K., Clark, D. S. & Dordick, J. S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1100–1104.
3. Shoichet, B. K., Baase, W. A., Kuroki, R. & Matthews, B. W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 452–456.
4. Malcolm, B. A., Wilson, K. P., Matthews, B. W., Kirsch, J. F. & Wilson, A. C. (1990) *Nature (London)* **345,** 86–89.
5. Popot, J.-L. & Engelman, D. M. (1990) *Biochemistry* **29,** 4031–4037.
6. Baker, D. & Agard, D. A. (1994) *Biochemistry* **33,** 7505–7509.
7. Veatch, W. R., Fossel, E. T. & Blout, E. R. (1974) *Biochemistry* **13,** 5249–5256.
8. Bystrov, V. F., Arseniev, A. S., Barsukov, I. L. & Lomize, A. L. (1987) *Bull. Magn. Reson.* **8,** 84–94.
9. Bano, M. deC., Braco, L. & Abad, C. (1989) *FEBS Lett.* **250,** 67–71.
10. Pascal, S. M. & Cross, T. A. (1992) *J. Mol. Biol.* **226,** 1101–1109.
11. Killian, J. A., Prasad, K. U., Hains, D. & Urry, D. W. (1988) *Biochemistry* **27,** 4848–4855.
12. LoGrasso, P. V., Moll, F., III & Cross, T. A. (1988) *Biophys. J.* **54,** 259–267.
13. Ketchem, R. R., Hu, W. & Cross, T. A. (1993) *Science* **261,** 1457–1460.
14. Cross, T. A. & Opella, S. J. (1994) *Curr. Opin. Struct. Biol.* **4,** 574–581.
15. Ulrich, A. S., Watts, A., Wallat, I. & Heyn, M. P. (1994) *Biochemistry* **33,** 5370–5375.
16. Smith, R., Separovic, F., Milne, T. J., Whittaker, A., Bennett, F. M., Cornell, B. A. & Makriyannis, A. (1994) *J. Mol. Biol.* **241,** 456–466.
17. Koeppe, R. E., II, Killian, J. A. & Greathouse, D. V. (1994) *Biophys. J.* **66,** 14–24.
18. Shon, K., Kim, Y., Colnago, L. A. & Opella, S. J. (1991) *Science* **252,** 1303–1305.
19. Fields, C. G., Fields, G. B., Noble, R. L. & Cross, T. A. (1989) *Int. J. Pept. Protein Res.* **33,** 298–303.
20. Pascal, S. M. & Cross, T. A. (1993) *J. Biomol. NMR* **3,** 495–513.
21. Langs, D. A. (1988) *Science* **241,** 188–191.
22. Nicholson, L. K. & Cross, T. A. (1989) *Biochemistry* **28,** 9379–9385.
23. Douliez, J. P., Leonard, A. & Duforc, E. J. (1995) *Biophys. J.* **68,** 1727–1739.
24. White, S. H. & Wiener, M. C. (1995) in *Permeability and Stability of Lipid Bilayers*, eds. DiSalvo, E. A. & Simon, S. A. (CRC, Boca Raton, FL), pp. 1–19.
25. Urry, D. W., Long, M. M., Jacobs, M. & Harris, R. D. (1975) *Ann. N.Y. Acad. Sci.* **264,** 203–220.
26. Salemma, F. R. (1988) *Science* **241,** 229–230.
27. Wallace, B. A. (1990) *Annu. Rev. Biophys. Biophys. Chem.* **19,** 127–157.
28. O'Connell, A. M., Koeppe, R. E., II & Andersen, O. S. (1990) *Science* **250,** 1256–1259.
29. Zhang, Z., Pascal, S. M. & Cross, T. A. (1992) *Biochemistry* **31,** 8822–8828.
30. Schroeder, F. & Nemecz, G. (1990) in *Advances in Cholesterol Research*, eds. Esfahani, M. & Swaney, J. (Telford Press, West Caldwell, NJ), pp. 47–87.
31. Rodrigueza, W. V., Wheeler, J. J., Klimuk, S. K., Kitson, C. N. & Hope, M. J. (1995) *Biochemistry* **34,** 6208–6217.