Importance of Tryptophan Dipoles for Protein Function: 5-Fluorination of Tryptophans in Gramicidin A Channels

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Abstract: In integral membrane proteins the amphipathic aromatic amino acid residues tryptophan and tyrosine tend to be localized at membrane/solution interface. The interfacial location of these residues is likely to be significant for membrane protein structure and function. Trp and Tyr have complex chemical characteristics, however, and it is difficult to deduce how these side chains determine protein structure and function. Specifically, Trp and Tyr not only are amphipathic but also dipolar, and electrostatic interactions that involve the side chain dipoles could be important for function. We evaluate the importance of the Trp dipole moment for ion channel function by replacing Trp residues in gramicidin A by the more polar 5-F-Trp and monitoring the ensuing changes in the conductance of membrane-spanning gramicidin channels. Trp → 5-F-Trp substitutions increase the conductance of the sequence-substituted channels, and we conclude that Trp side chains increase ion permeability through electrostatic interactions with the permeant cations.

Introduction

Among the 20 common amino acids, tryptophan, tyrosine, and phenylalanine are particularly enigmatic because their side chains have rigid aromatic rings, which are at once hydrophobic and polar. The π-electron systems of the rings enable the side chains to solvate cations and be hydrogen bond acceptors.[1,2] The side chain rigidity also will decrease the conformational entropy penalty for noncovalent interactions of these amino acid side chains with their surroundings (cf. ref 3). In addition, the NH and OH moieties of Trp and Tyr serve as hydrogen bond donors and endow the side chains with permanent dipole moments. This unique combination of properties allows Trp and Tyr (and to a lesser extent Phe) to participate favorably in an unusually wide range of intermolecular and intramolecular interactions;[2,3] it also means that there is considerable divergence about where to place Trp and Tyr on hydrophobicity scales.[4−11] The aromatic amino acids also may play a special role in assembling partially folded, stable structures early in protein folding.[12]

In membrane proteins, Trp and Tyr tend to cluster near the membrane/solution interface within the transmembrane region.[13−18] This interfacial localization, as well as the importance of aromatic amino acids for protein function, may arise because Trp and Tyr not only are hydrophobic but also are able to form hydrogen bonds and possess dipole moments. Examination of the porin structure[14,15] shows a striking difference in the organization of Trp and Tyr on one hand and Phe on the other: Trp and Tyr side chains tend to project toward the aqueous solution; Phe side chains tend to project toward the membrane interior. This difference in organization most likely reflects the characteristic differences between the side chains of Trp and Tyr as compared to Phe. The organization of Trp and Tyr has at least two consequences: the NH and OH moieties can form hydrogen bonds to polar groups at the membrane/solution interface; and the side chain dipole moments are aligned, which will establish a local modification of the interfacial dipole potential that can affect the electrostatic potential profile within the protein (cf. ref 19). Such effects can be significant because the dipoles are oriented in a relatively low dielectric constant medium (cf. refs 20−22).

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Are the properties of hydrogen bond propensity and dipole moment separable? We address here this fundamental question, and show that it is possible to determine if a particular function of Trp should be ascribed to the indole ring's dipole moment as opposed to its ability to form hydrogen bonds. (Hydrogen bonds between the indole NH and H_2O, or other hydrogen acceptors at the membrane/solution interface, also possess a dipole moment (cf. ref 23). In the present experiments, however, we investigate only the dipole moment associated with the indole ring proper.)

To approach this problem, it is helpful to use a defined system in which tryptophan indole rings are held in a rather fixed geometry at the membrane/solution interface of a phospholipid bilayer. For our experiments, we use the gramicidin A (gA) $\beta^{6.3}$-helical channel,[24] which serves as a framework for holding the rings in place and for ascertaining their functional importance. The sequence of a gA monomer is[25] formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp^9-D-Leu-L-Trp^{11}-D-Leu-L-Trp^{13}-D-Leu-L-Trp^{15}-ethanolamine. Dimers of gA span lipid bilayer membranes and permit single-file cation conductance at very high rates—so high that they allow one to estimate the heterogeneous collision velocity for hydrated ions in aqueous solution.[26] The four Trp residues are oriented such that the indole NH moieties point out, toward the aqueous phase.[27−29] The gA single-channel conductance depends critically on these four Trp;[30−32] replacement of any one of them with Phe reduces the single-channel conductance by about 20−30%.[33] These results have been interpreted in terms of through-space (attractive) electrostatic ion−dipole interactions by which the indole rings promote cation flux through the channel. (Similar, but repulsive, ion−dipole interactions have been found when aromatic side chains are substituted at position one in gA.[36]) Trp → Phe substitution experiments, however, cannot definitively distinguish single-channel conductance changes due to electrostatic interactions from changes due to the removal of a hydrogen bond, which could alter the side chain localization and thus the channel function. A structural role of the Trp residues is, in fact, suggested by the observation that removal of Trp^{15} decreases the single-channel conductance 4-fold.[37]

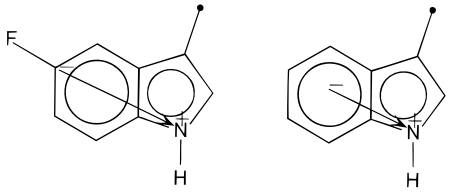

Figure 1. Chemical structures of the Trp (left) and 5-F-Trp (right) side chains. Each arrow indicates the approximate direction and magnitude of the dipole moment, 2.1 D for indole and 3.6 D for 5-F-indole.[38]

In the present experiments, we maintained the hydrogen bond-forming ability of the indole group as well as the direction of the indole dipole, while at the same time enhancing the magnitude of the dipole moment by introducing a fluorine at position 5 in the indole side chain. The resulting 5-fluoro-tryptophan (5-F-Trp) is structurally similar to Trp, and their dipole moments have similar orientations but different magnitudes:[38] 3.6 D for 5-F-indole compared to 2.1 D for indole (Figure 1). We introduced 5-F-Trp at one of two positions (either 9 or 11) in gA. To optimize the detection of conductance changes that result from a Trp → 5-F-Trp substitution, the other Trp (either 11 or 9) was replaced by Phe, to lower the conductance of the reference channel.[33]

As an added feature, the 5-F-Trp-containing gA analogues were synthesized by incorporation of enantiomeric 5-F-(D,L)-Trp. The resulting diastereomers were separated chromatographically, the channel-forming ability of each was tested, and the single-channel conductance of the active 5-F-(L)-Trp diastereomer was compared to that of the equivalent nonfluorinated gramicidin. We thus were able to monitor the effect of enhancing a single side chain dipole moment, by a single fluorine atom, upon the gA single-channel conductance.

Results

Separation and Identification of Diastereomers. Figure 2a shows the separation of [5-F-(D,L)-Trp^9,Phe^{11}]gA by reversed-phase high-performance liquid chromatography. There are two clearly defined peaks in the chromatogram. The compound with 5-F-(L)-Trp^9 has the proper alternating L,D chirality throughout the sequence, which allows it to fold properly and have fairly strong interactions with the stationary phase. Consequently, one would expect [5-F-(L)-Trp^9,Phe^{11}]gA to be the second to elute. Conversely, a D-residue at position 9 will interrupt the alternating L,D pattern, which will cause improper folding and weaker interaction with the C8 stationary phase; the compound with 5-F-(D)-Trp^9, therefore, should elute early.

To verify this a priori expectation, the contents of the peaks in Figure 2a were examined electrophysiologically. With material from the first HPLC peak, there is no current activity at even the maximal aqueous gramicidin concentration used (3 nM) (Figure 2b). This result confirms that the peptide in the first peak is improperly folded due to the presence of 5-F-(D)-Trp^9. With material from the second HPLC peak, there is well-defined channel activity at picomolar concentrations (Figure 2c). That is, the material in HPLC peak #2 represents the bona fide [5-F-(L)-Trp^9,Phe^{11}]gA, and the inactive isomer with the improper backbone-folding does indeed elute earlier. Similarly, we purified [Phe^9,5-F-(L)-Trp^{11}]gA from the mixture of diastereomers and definitively identified the product by single-channel analysis. Again, the inactive isomer eluted first, and the properly folded and channel-forming isomer eluted second (results not shown).

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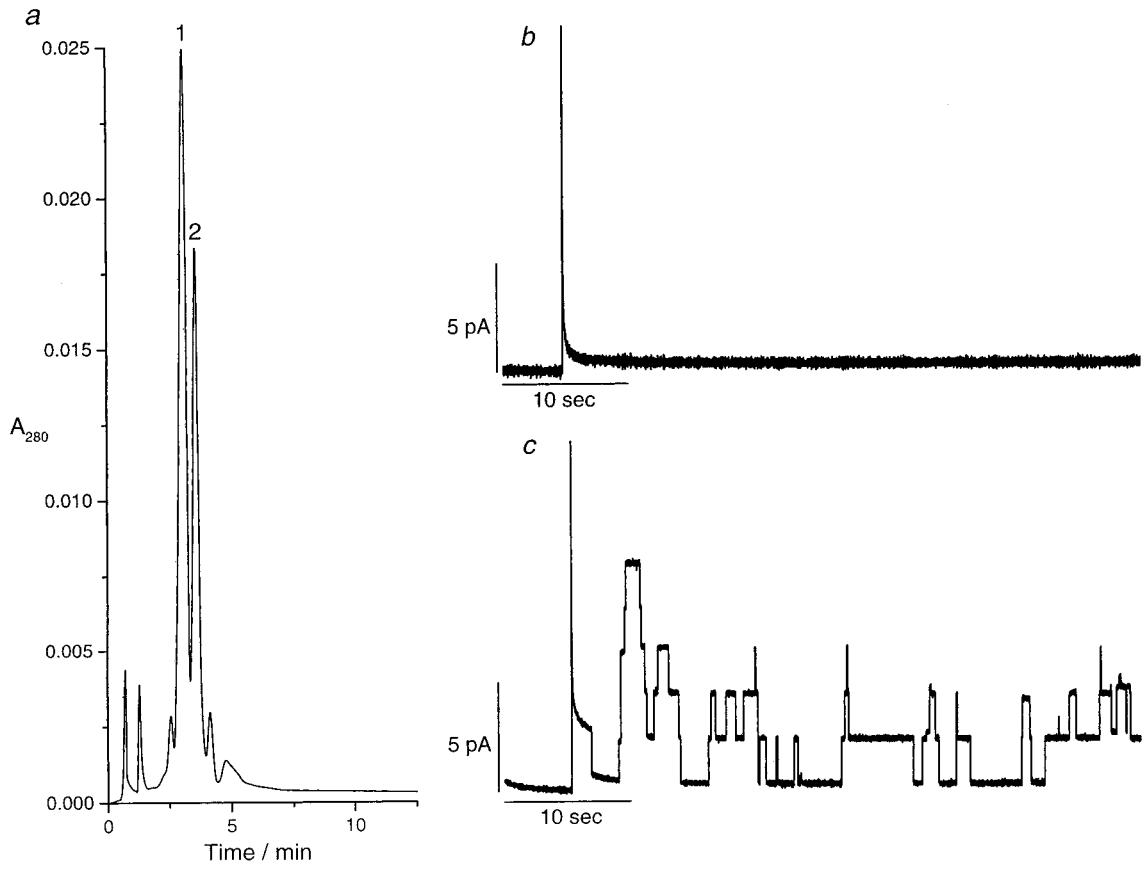

Figure 2. Separation and single-channel analysis of [5-F-D-Trp^9,Phe^{11}]gA and [5-F-L-Trp^9,Phe^{11}]gA. (a) HPLC trace obtained with the original synthesis, before separating the L-(5-F-Trp)- and the D-(5-F-Trp)-containing gramicidin analogues. There are two peaks labeled 1 and 2, respectively. (b) Current trace obtained with 3 nM of the material in peak #1 to both sides of a diphytanoylphosphatidylcholine (DPhPC)/*n*-decane bilayer. The calibration bars denote 10 s (horizontally) and 2 pA (vertically). (c) Current trace obtained with 30 pM of the material in peak #2 to both sides of a DPhPC/*n*-decane bilayer. Calibrations as in (b).

Single-Channel Properties. Trp → 5-F-Trp substitutions in gA alter both the conductance and the average duration of gramicidin channels in planar lipid bilayers (Figure 3).

Figure 3 shows current traces for channels formed by the fluorinated and reference gramicidins (from top to bottom): gA, [Phe^{11}]gA, [5-F-Trp^9,Phe^{11}]gA, [Phe^9]gA, and [Phe^9,5-F-Trp^{11}]-gA.

Figure 4 shows current transition amplitude and channel lifetime histograms for channels formed by the fluorinated analogues [5-F-Trp^9,Phe^{11}]gA and [Phe^9,5-F-Trp^{11}]gA.

Table 1 summarizes information on a larger series of analogues. Substitution of any of the Trp residues by Phe decreases the single-channel conductance, and the introduction of two Phe's reduces the conductance still further. That the conductance decrements are due to the loss of one or two Trp dipoles is confirmed by the Trp → 5-F-Trp substitutions. The conductance is enhanced 30−60% when 5-F-Trp is introduced at either position 9 (in [Phe^{11}]gA) or position 11 (in [Phe^9]gA).

Discussion

The gramicidin transmembrane channel fixes four tryptophans at the membrane/water interface, in orientations that are well-defined.[28,29,39] The present results, in which 5-fluorination of either of the two different tryptophans directly amplifies the single-channel conductance, serve to clarify and extend earlier findings with Phe-substituted gramicidins[30−34] and strongly suggest that a consistent electrostatic mechanism is sufficient to explain all of the data.

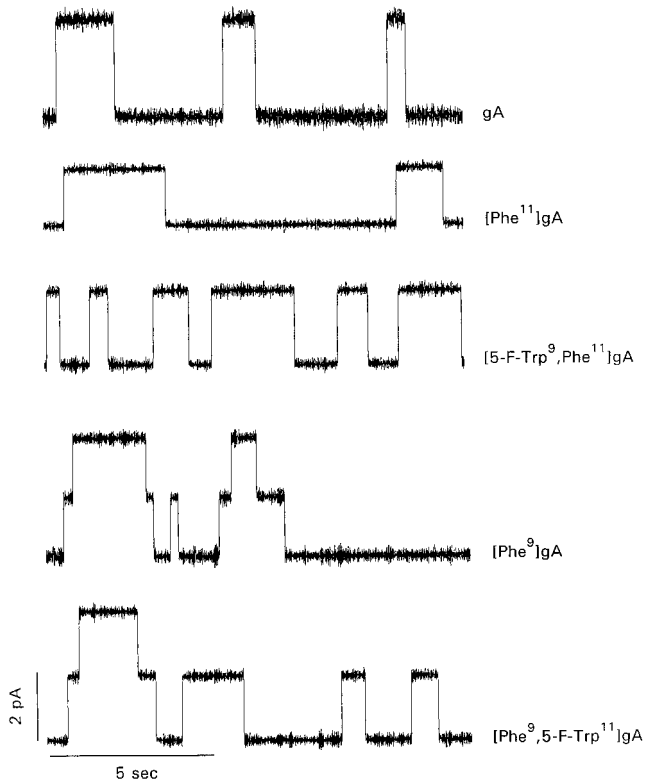

Figure 3. Single-channel current traces for channels formed by (from top to bottom) gA, [Phe^{11}]gA, [5-F-Trp^9,Phe^{11}]gA, [Phe^9]gA, and [Phe^9,5-F-Trp^{11}]gA.

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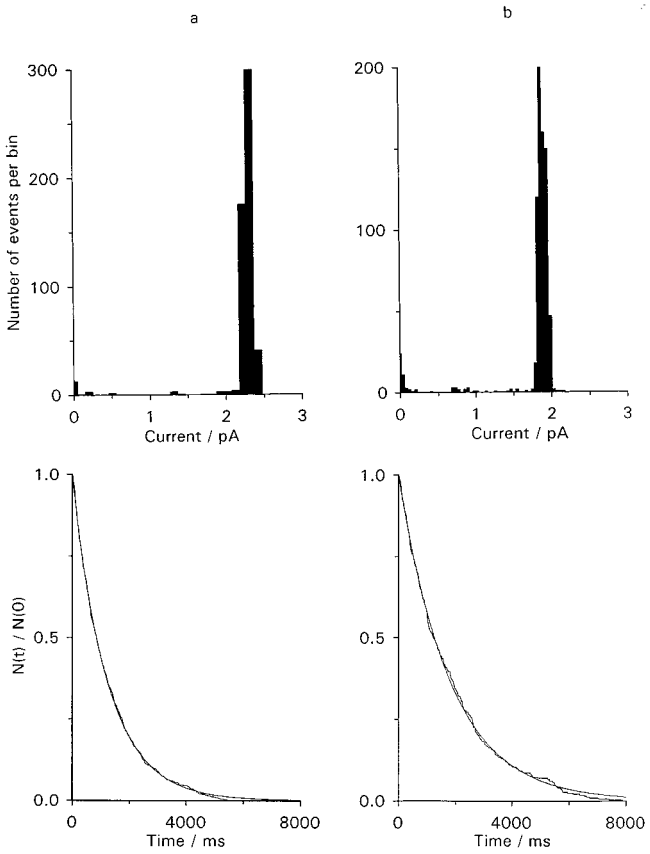

Figure 4. Current transition amplitude histograms and lifetime distributions for [5-F-Trp^9,Phe^{11}]gA (a) and [Phe^9,5-F-Trp^{11}]gA (b).

Table 1. Single-Channel Conductances and Average Lifetimes[a]

gramicidin analogue	conductance[b] (pS)	lifetime (ms)
gA	15.0 ± 0.3	620
[Phe^9]gA[c]	6.0 ± 0.1	1000
[5-F-Trp^{11},Phe^9]gA	9.7 ± 0.3	1800
[Phe^{11}]gA[c]	8.7 ± 0.3	2300
[5-F-Trp^9,Phe^{11}]gA	11.2 ± 0.6	980
[Phe^{13}]gA[c]	11.2 ± 0.1	800
[Phe^{15}]gA[c]	10.9 ± 0.1	790
[Phe^{9,15}]gA[c]	4.1 ± 0.1	750
[Phe^{11,15}]gA	3.3 ± 0.2	830

[a] 1.0 M NaCl, 200 mV, 25 ± 1 °C. [b] Mean ± standard deviation. [c] From ref 33.

Trp → Phe Substitutions. Kinetic analysis of Na^+ permeation through Trp → Phe substituted gramicidin channels shows that these substitutions alter all rate constants involved in the ion transfer process.[34] The effects of Trp → Phe substitutions on the dynamics of ion entry and exit are difficult to interpret, because the different chemical properties of the side chains may alter their short-range interactions with other groups at the membrane/solution interface. The present experiments therefore were done at relatively high permeant ion concentrations (1.0 M NaCl), where the major resistance to ion movement through the channel is the electrodiffusive movement through the pore (cf. ref 34). The effects (of a Trp → Phe substitution) on the energy barrier for ion movement through the central part of the pore could suggest that long-range electrostatic interactions were involved; such an interpretation would be consistent with earlier work that demonstrated the importance of dipolar residues at position 1.[40,41] Nevertheless, Trp → Phe substitutions alter other side chain properties, with the most notable being the indole NH group that can form hydrogen bonds to polar groups at the membrane/solution interface,[42,43] and the reduction in aromaticity when going from a two-ring 10-electron π system to a single-ring 6-electron π system. Not surprisingly, experiments with other side chains substituted in place of the four Trp residues have been ambiguous regarding the importance of ion−dipole interactions,[31] and analogues in which all four Trp residues have been replaced by Phe exhibit different folding preferences.[44,45]

The Trp → Phe results previously have been interpreted to be due to favorable electrostatic interactions between the indole side chains and the permeating ions.[34,46] Trp → Phe experiments, however, cannot distinguish whether changes in conductance should be attributed to electrostatic interactions, to the missing hydrogen bond, or even to the altered aromaticity (or any combination of these effects).

Electrostatic Mechanism. The results with Trp → 5-F-Trp substitutions resolve this ambiguity—because the 10-electron aromaticity and the indole NH moiety are retained in 5-F-Trp, because the channel folding is not affected (as determined in heterodimer formation experiments, results not shown), and because the average side chain orientation is unaffected by this substitution, as indicated by solid-state nuclear magnetic resonance experiments.[47] In support of the electrostatic interpretation, the introduction of a 5-F substitution on either Trp^9 or Trp^{11} increases the single-channel conductance by 30−60% (Table 1), under conditions where the hydrogen bonding should remain intact.

Our results show that the interfacial indole dipoles determine the magnitude of the single-channel conductance according to a fairly simple scheme: the removal of a side-chain dipole—by a Phe substitution—diminishes the single-channel conductance,[33] while the enhancement of a side chain dipole by 5-fluorination increases the conductance. Solid-state NMR results show that the indole dipoles in gA channels are oriented such[28,39,46] that they can lower the central barrier for ion movement through the channel and thus help attract cations into the channel.[46,48] Figure 5 shows a schematic representation of the dipole moment vector ($\mu_{C_5\rightarrow N}$) in Trp^{11}: its projections to planes that are parallel ($\mu^{||}_{C_5\rightarrow N}$) and perpendicular ($\mu^{\perp}_{C_5\rightarrow N}$) to the pore axis; the decomposition into components that are parallel ($\mu_{||}$), perpendicular ($\mu_{\perp}$), and tangential (μ_t) to the pore axis; the projection directed toward the channel center (μ_{cntr}), cf. refs 40 and 46. The direction of μ_{cntr} is such that the replacement of Trp^{11} by Phe would be expected to increase the barrier for ion movement through the pore. (The favorable orientation of μ_{cntr} is due to $\mu_{||}$, whereas the $\mu_{\perp}$ component is slightly repulsive.) Conversely, a Trp → 5-F-Trp substitution, which will increase the magnitude of μ_{cntr}, would be expected to lower the barrier for ion movement through the pore—and thus increase the single-channel conductance. The electrostatic mechanism therefore can rationalize the conductance changes observed with both types of Trp modifications.

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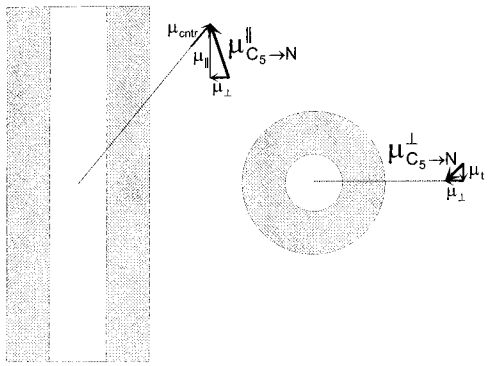

Figure 5. Schematic illustration of the orientation of the Trp, or 5-F-Trp, dipole moment ($\mu_{C_5\rightarrow N}$) and its decomposition into components relative to the pore axis of a membrane-spanning gA channel (drawn using Trp[11] coordinates from ref 28). $\mu^{\parallel}_{C_5\rightarrow N}$ (left) and $\mu^{\perp}_{C_5\rightarrow N}$ (right) denote projections of $\mu_{C_5\rightarrow N}$ onto planes that are parallel and perpendicular to the pore axis, respectively; $\mu_{\parallel}$, $\mu_{\perp}$, and μ_t denote the decomposition of $\mu_{C_5\rightarrow N}$ into components that are parallel, perpendicular, and tangential to the pore axis (the perpendicular and tangential components are relative to a line from the pore axis to the indole N atom); and μ_{cntr} denotes the projection of $\mu_{C_5\rightarrow N}$ along a line from the pore center to the indole N. The hatched areas represent the peptide backbone. $\mu_{C_5\rightarrow N}$ is drawn such that it spans the distance from C_5 to N; the arrowheads denote the positive end of the dipole vectors. On the basis of the Trp ring orientations in ref 28 or 46, the magnitude of the dipole moment components for Trp[9] are (in Debye, values for 5-F-Trp in parentheses) $\mu_{\parallel} = 1.2$ or 1.9 (2.1 or 3.3), $\mu_{\perp} = 0.05$ or 0.3 (0.1 or 0.5), and $\mu_{cntr} = 0.8$ or 1.6 (1.4 or 2.7). Similarly, for Trp[11]: $\mu_{cntr} = 1.9$ or 2.0 (3.3 or 3.4); $\mu_{\perp} = 0.7$ or 0.3 (1.2 or 0.5); $\mu_{cntr} = 1.0$ or 1.8 (1.8 or 3.1).

A similar simplicity does not hold in the case of the channel lifetimes, as the effects of the 5-F substitutions on side chains 9 and 11 have opposite effects on the average channel lifetimes (Table 1). This result agrees with previous results in which a series of Trp → Phe substitutions yielded widely varying effects on the channel lifetime.[33] Trp dipoles do not govern channel lifetimes according to a simple pattern (see also refs 32 and 49).

Electrostatic interactions between side chain dipoles and charged substrates may be important for globular proteins in general, e.g., acetylcholinesterase, where the active site is at the bottom of a "gorge" that is lined by a large number of aromatic residues[50] and electrostatic interactions between the substrate acetylcholine and a Trp in the active site are important for enzyme function [51]

Implications for Membrane Proteins. The aromatic residues are likely to promote the formation of nucleation sites in protein folding[3,12] and to stabilize the conformations and orientations of proteins within membranes.[16,43−45,52] The structural and mechanistic bases for these effects remain poorly understood. In the present experiments we enhanced the side chain dipole moment while retaining the indole NH moiety, which is likely to be important for the side chain orientation and mobility. The results complement previous experiments on Trp → aromatic residue substitutions and provide now direct support for the importance of ion−dipole interactions for ion permeation. Similar electrostatic interactions between the aromatic side chains at the membrane/solution interface and the remaining structure of integral membrane proteins may be important not only for ion permeability but also for other functions. Furthermore, the dipolar Trp residues may interact electrostatically with the dipoles of the phospholipid backbone and headgroup,[20] which have an orientation that is opposite to that of the indole NH-anchored Trp residues. Such interactions could allow for an efficient coupling of protein conformational changes to alterations in the packing of the bilayer lipids.[53]

Methods

Peptide Synthesis and Purification. (D,L)-5-Fluoro-tryptophan was purchased from Sigma Chemical Co. (St. Louis, MO). It was converted to the Fmoc derivative and incorporated into the gramicidins by total synthesis.[54] The diastereomeric peptide products were purified by reversed-phase chromatography using a 4.6 × 250 mm column of 5 μm octyl-silica, eluted with 82% methanol, 18% water at 1.5 mL/min, 1800 psi, 23 °C.[33,54]

Electrophysiology. Single-channel measurements were done using the bilayer punch[55] at 25 ± 1 °C using a Dagan 3900 patch-clamp amplifier (Dagan Instruments, Minneapolis, MN). The electrolyte solution was 1 M NaCl. The gramicidin analogues were added from an ethanolic stock. The applied potential was 200 mV. The current signal was filtered at 100 Hz, digitized, and sampled at 500 Hz using a PC/AT compatible computer; transitions were detected on-line;[55] and single-channel current transition amplitudes and lifetimes were determined as described previously[56] with software written using AxoBasic (Axon Instruments, Foster City, CA).

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