Chapter 14
Molecular Anatomy of an Ion Channel Explored Utilizing Fluorescence Spectroscopy

Arunima Chaudhuri and Amitabha Chattopadhyay

Abstract Ion channels are transmembrane proteins and represent important cellular components that connect the inside of the cell to its outside in a selective fashion. The linear ion channel peptide gramicidin serves as an excellent prototype for monitoring the organization, dynamics and function of membrane-spanning channels due to a variety of reasons. The fluorescent tryptophan residues in gramicidin channels are crucial for establishing and maintaining the structure and function of the channel in the membrane bilayer. In this review, we have highlighted a variety of representative fluorescence-based approaches to gain molecular insight into gramicidin conformations. Since gramicidin shares common structural features with more complex ion channels, the results from fluorescence-based studies with gramicidin could be relevant for more complex ion channels.

Keywords Ion channel • Gramicidin • REES • Tryptophan • Membrane interface

14.1 Introduction

Ion channels are important cellular nanomachines that regulate ionic permeability in cell membranes. They are integral membrane proteins with multiple transmembrane domains. Their ability to connect the inside of the cell to its outside in a selective fashion makes them crucial elements in cellular signaling and sensing. Defects in the function of ion channels result in diseases [1] such as cystic fibrosis [2]. Advances in DNA sequencing technology have linked many diseases to defects in ion channels and, the term 'channelopathy' has been coined [3]. Drugs acting on ion channels have long been used as therapeutics for treatment of a wide spectrum of disorders. This makes them a favorite target for the pharma industry and ~15 % of the world's 100 top-selling drugs are currently targeted to ion channels [4].

Although ion channels are important members in cellular physiology, detailed structure-function analysis of ion channels at high resolution has proved to be

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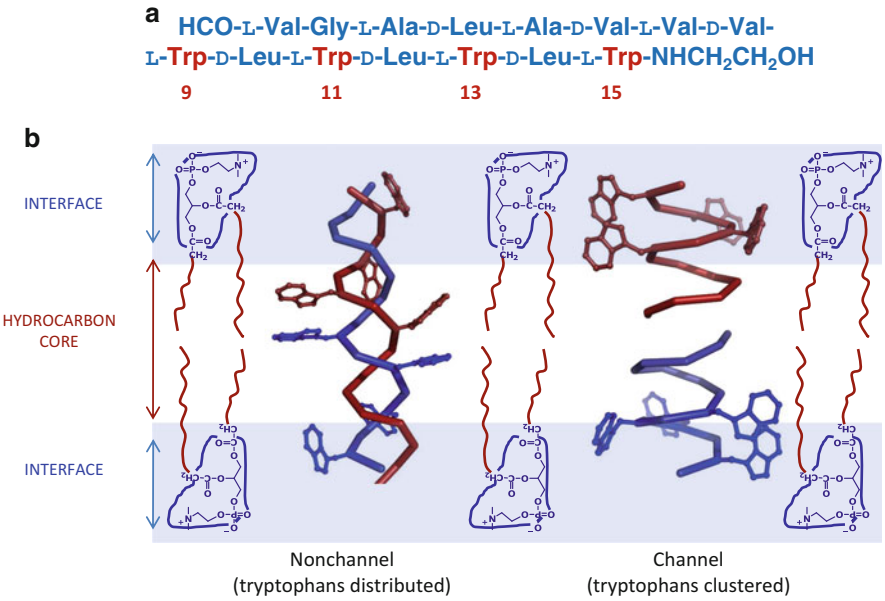

Fig. 14.1 (**a**) The amino acid sequence of gramicidin A with its unique alternating L- and D-chirality. Aromatic amino acids (tryptophans) are highlighted and their positions are indicated. (**b**) A schematic representation of the two predominant forms of gramicidin, the nonchannel and channel conformations, displaying the locations of tryptophan residues in the membrane bilayer. A hallmark of the channel conformation is the clustering of tryptophans toward the membrane interface. A distinctive difference is observed in the tryptophan distribution in the nonchannel conformation in membranes, where the tryptophans span the entire bilayer normal. See text for other details (Adapted and modified from refs. [5] and [26] with permission from Elsevier)

challenging till very recently. In the overall context of the complexity and difficulty involved in studying large ion channels at molecular resolution, the linear peptide gramicidin has proved to be a relevant model for ion channels. Gramicidin forms prototypical ion channels specific for monovalent cations and has been extensively used to monitor the organization, dynamics, and function of membrane-spanning channels and integral membrane proteins [5]. Gramicidin is a multi-tryptophan peptide (Trp-9, 11, 13, and 15) with alternating L- and D-chirality (see Fig. 14.1a). The advantages of gramicidin as ion channel include its small size, ready availability and the relative ease with which chemical modifications can be performed. These excellent features contribute to the usefulness of gramicidin and form the basis for its use to explore the principles that govern the folding and function of ion channels.

More importantly, gramicidin channels share important structural features involving ion selectivity with complex ion channels such as KcsA potassium channels [6]. The unique sequence of alternating L- and D-chirality allows gramicidin to assume a variety of environment-sensitive conformations. Among these, two major conformations are: (i) the single stranded $\beta^{6.3}$ helical dimer (the 'channel' form), and (ii) the double stranded intertwined helix (collectively known as the

'nonchannel' form) (see Fig. 14.1b) [5]. The amino terminal-to-amino terminal single-stranded $\beta^{6.3}$ helical dimer form is the thermodynamically preferred conformation in membranes and membrane-mimetic media. In this conformation, the tryptophan residues remain clustered at the membrane-water interface [7–10]. Interestingly, the membrane interfacial localization of tryptophan residues is absent in 'nonchannel' conformations and the tryptophan residues are distributed along the membrane axis [5, 7, 11]. Nonchannel conformations have been shown to exist in membranes with polyunsaturated lipids [12], and in membranes with increased acyl chain lengths under hydrophobic mismatch conditions [13, 14].

14.2 Tryptophan: A Uniquely Placed Amino Acid in Membrane Proteins

The presence of tryptophan residues as intrinsic fluorophores in membrane peptides and proteins makes them an attractive choice for fluorescence spectroscopic analyses [15–19]. Tryptophan residues play an important role in the structure and function of membrane proteins and peptides (recently reviewed in ref. 19). Tryptophan residues in membrane proteins and peptides are not uniformly distributed, but tend to be localized toward the membrane interface (see Fig. 14.2). The interfacial region in membranes has unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane [18, 20, 21]. The interfacial localization of tryptophan in membrane proteins and peptides, along with the fact that the distribution and localization of tryptophans in the two major conformations of gramicidin are distinctly different (see Fig. 14.1b), allow us to explore conformations adopted by gramicidin and its tryptophan analogs using fluorescence spectroscopic readouts. In this review, we have highlighted representative fluorescence-based approaches to gain insight into gramicidin conformations. Since gramicidin is a prototypical ion channel [5] and shares common structural features with more complex ion channels, which are more challenging to work with [6], the results from these studies form the basis of addressing ion channel conformations under varying conditions.

14.3 Ion Channel Conformations Explored Using REES

Red edge excitation shift (REES) is a popular tool to explore organization, dynamics and conformation of membrane probes, proteins and peptides [17–20]. REES is defined as the shift in the wavelength of maximum fluorescence emission toward longer wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption spectrum. REES becomes significant in case of fluorophores with a relatively large change in dipole moment upon excitation in restricted

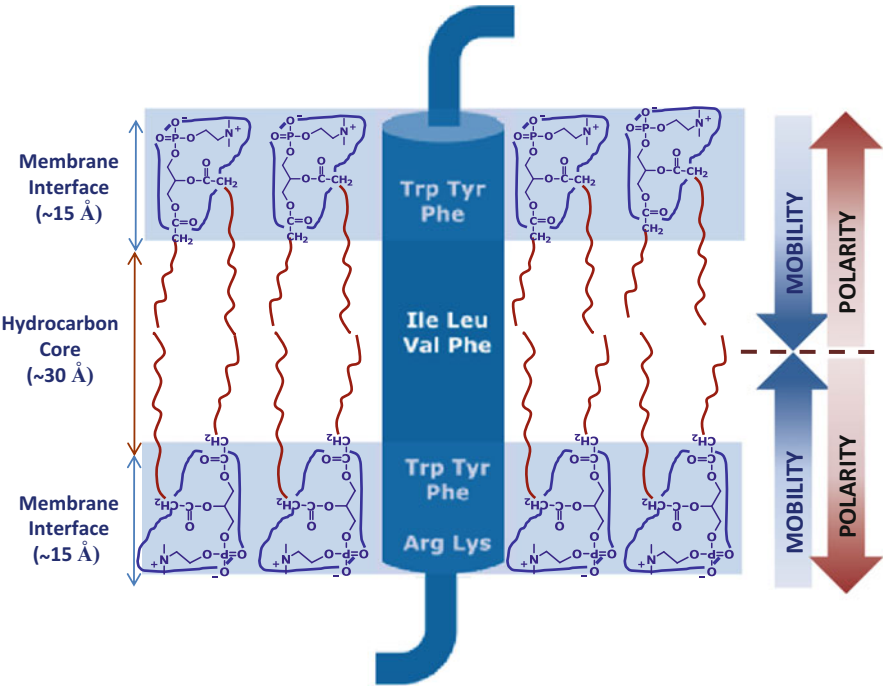

Fig. 14.2 A schematic representation of a typical transmembrane domain of a representative membrane protein (such as ion channels) in the bilayer showing distinct preferences of various amino acids for different parts of the membrane bilayer. The membrane lipids shown have two hydrophobic tails with a phosphatidylcholine headgroup. It should be noted that the aromatic amino acids, especially tryptophan and tyrosine residues, are localized in the membrane interface region, a feature shared by many ion channels [11]. The membrane interface, constituting ~ 50 % of the thickness of the bilayer, is represented by a heterogeneous environment characterized by relatively slow dynamics. This region also exhibits higher polarity relative to the hydrophobic core, predominantly due to the restricted water molecules (Adapted and modified with permission from ref. [19] (copyright (2014) American Chemical Society))

environment. Unlike other fluorescence approaches, REES data contain information on the rotational dynamics of excited state dipoles around the fluorophore, thereby providing a window to the dynamics of the environment. In our laboratory, we have utilized REES to monitor the conformation and dynamics of a variety of membrane peptides and proteins including gramicidin [7, 10, 22–26], melittin [27, 28], the pore-forming α-toxin from *S. aureus* [29], the N-terminal domain of CXC chemokine receptor (CXCR1) [30], and membrane-bound bovine α-lactalbumin. [31] A particularly attractive example is the application of REES to conformational analysis of gramicidin, a representative ion channel peptide.

As mentioned above, two major conformations adopted by gramicidin in various media are: (i) the single stranded $\beta^{6.3}$ helical dimer (the 'channel' form), and (ii) the double stranded intertwined helix (collectively known as the 'nonchannel' form) (see Fig. 14.1b). In the channel conformation in membranes, the tryptophan

residues are clustered at the membrane-water interface [7, 9, 10]. We earlier showed that the tryptophan residues of gramicidin in the channel conformation exhibit REES, implying that the tryptophan residues are localized in the interfacial region and experience motional restriction [7, 10]. In an important application of REES to conformational analysis of membrane proteins, we demonstrated that various conformations of membrane-bound gramicidin could be distinguished using their REES signatures (see Fig. 14.3) [7]. The basic principle of such conformation-specific REES is the fact that the microenvironment of the tryptophans in these conformations are different, thereby giving rise to different REES readouts (see Fig. 14.3b). For example, while the tryptophan residues are clustered toward the membrane interface in the channel conformation of gramicidin, they are distributed along the membrane axis in the nonchannel conformation. We have previously demonstrated, using anthroyloxy probes, that the vertical location (depth) of a fluorophore in the membrane is an important parameter in their ability to exhibit REES [32]. Fluorophores localized at the shallow membrane interfacial region experience restricted dynamics due to the physicochemical nature of the interfacial region, and exhibit REES. On the other hand, fluorophores localized at deeper locations in the membrane experience much more dynamic environment and exhibit reduced REES. This principle has proved to be very useful in conformational analysis of gramicidin in membranes. In agreement with this, the tryptophans in the channel conformation of gramicidin (with tryptophans at the motionally restricted interfacial region) give rise to REES of 7 nm. In contrast, tryptophans in the nonchannel conformation of gramicidin (distributed along the membrane axis with varying degrees of motional restriction) give rise to REES of only 2 nm. More importantly, it was possible to monitor REES exhibited by conformational intermediates in the folding pathway of membrane-bound gramicidin from the initial nonchannel to the final channel conformation (denoted as intermediates I and II in Fig. 14.3b). The progressive increase in REES from the nonchannel conformation to the channel conformation through the intermediate folding conformations corresponds to the conversion of the nonchannel form to the channel form of gramicidin. This is due to gradual change in the location of tryptophan residues from a distribution along the bilayer normal to being clustered at the membrane interface.

14.4 Conformational Heterogeneity: Use of Fluorescence Lifetime Distribution Analysis

Conformational heterogeneity in membrane proteins can be assessed using fluorescence lifetime distribution analysis of tryptophan residues by the maximum entropy method (MEM). MEM represents a model-free robust approach for analyzing fluorescence lifetime distribution [21, 33–35]. The width of the lifetime distribution obtained by this method is correlated with the degree of heterogeneity of the environment sensed by the fluorophore.

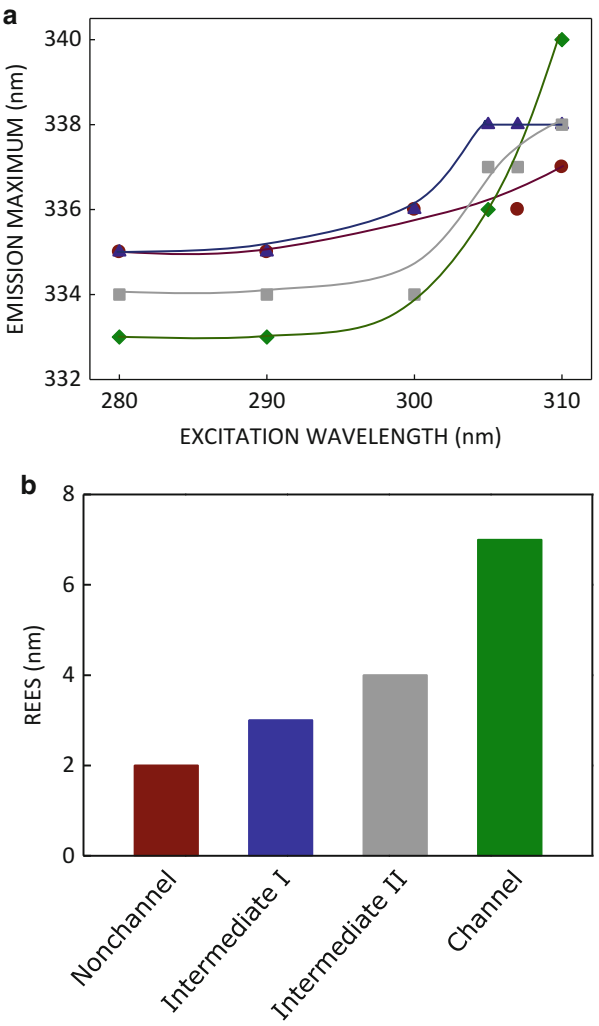

Fig. 14.3 Monitoring conformations of ion channels using REES as a reporter. (**a**) Effect of changing excitation wavelength on the wavelength of maximum emission for the nonchannel (●), intermediate I (▲), intermediate II (■), and channel (♦) forms of gramicidin. (**b**) The magnitude of REES is sensitive to the conformation of ion channel adopted by gramicidin. Intermediates I and II represent intermediates in the folding pathway from the nonchannel conformation to the channel conformation. The magnitude of REES corresponds to the total shift in emission maximum as the excitation wavelength was changed from 280 to 310 nm. The lines joining data points are provided merely as viewing guides (Adapted and modified with permission from ref. [7] with permission from Elsevier and ref. 19 (copyright (2014) American Chemical Society))

The differential conformational heterogeneity sampled by gramicidin conformers is shown in Fig. 14.4. The figure shows tryptophan lifetime distributions by MEM analysis in the channel and nonchannel conformations of gramicidin [36]. Interestingly, fluorescence lifetime distribution of tryptophan residues in the nonchannel form (characterized by a width of the distribution (w) of 3.40 ns, represented as full width at half maxima for the major lifetime peak) was found to be significantly broader relative to the corresponding width for the channel form ($w = 0.96$ ns). This indicates that the tryptophan residues in the nonchannel form experience relatively heterogeneous environment relative to the environment experienced in the channel form. This is in agreement with the fact that tryptophan residues are clustered at the membrane interfacial region in the channel form, while

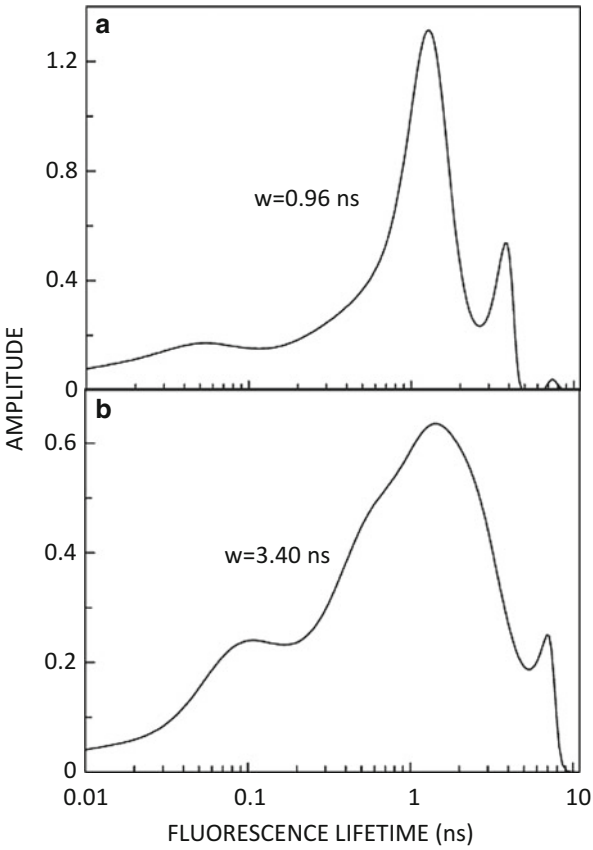

Fig. 14.4 Conformation-dependent heterogeneity profiles of the tryptophan residues in gramicidin derived from MEM lifetime distribution analysis for the (**a**) channel and (**b**) nonchannel conformations. Variation in the width of the tryptophan fluorescence lifetime distribution (w) for the major peaks indicates differential membrane heterogeneity experienced by the tryptophans localized at different regions of the membrane z-axis in the channel and nonchannel conformations (Reproduced from ref. [36] with kind permission from Springer Science and Business Media)

they are spread all across the bilayer normal in case of the nonchannel form (see Fig. 14.1b). Fluorescence lifetime distribution analysis by MEM therefore provides a novel window to monitor such conformational transitions in membrane proteins such as ion channels. Since ion channels require a variety of conformations for carrying out their function, this approach provides a unique way to sample the conformational plasticity associated with each conformation.

14.5 Understanding the Functional Role of Tryptophans in Gramicidin Channel: Insights from Tryptophan Analogs

Interestingly, tryptophans in gramicidin channels have been shown to be crucial for maintaining the structure and function of the channel [5]. The importance of gramicidin tryptophans is apparent from the observation that the cation

conductivity of the ion channel decreases upon substitution of one or all of the tryptophan residues by phenylalanine, tyrosine or naphthylalanine [37–39], and also upon ultraviolet irradiation or chemical modification of the tryptophan side chains [40–42]. Additionally, it has been shown that gramicidins with Trp → Phe substitutions face greater difficulty in forming membrane-spanning dimeric channels [39, 43]. With an overall goal of understanding the structural basis of the role of tryptophans in maintaining the ion channel structure of gramicidin, we used single tryptophan gramicidin analogs with three Trp → Ser-*t*-butyl substitutions using a combination of fluorescence approaches which include REES and membrane penetration depth analysis [24]. The Ser-*t*-butyl side chain was chosen because it is approximately as hydrophobic as Phe, yet not aromatic. Figure 14.5a shows the amino acid sequence of these single tryptophan analogs. REES results using these single tryptophan analogs are shown in Fig. 14.5 (panels b and c). Figure 14.5c shows that single tryptophan analogs Trp-15, Trp-13, and Trp-11 exhibited REES of 4–7 nm. In contrast, Trp-9 displayed enhanced REES of 15 nm. Based on these results in combination with membrane penetration depth analysis [44], size-exclusion chromatography and backbone CD data, we demonstrated that the gramicidin analogs containing single tryptophan residues adopt a mixture of channel and nonchannel conformations, thereby reducing ion channel activity.

In order to address the basis of differential importance of tryptophan residues in gramicidin channel, we explored the effects of pairwise substitution of two of the four gramicidin tryptophans, the inner pair (Trp-9 and -11) and the outer pair (Trp-13 and -15), using a combination of steady state and time-resolved fluorescence approaches and circular dichroism spectroscopy (see Fig. 14.6a for the sequence of double tryptophan analogs) [25]. The normalized emission spectra of the double tryptophan gramicidin analogs ($Phe^{9,11}$gA and $Phe^{13,15}$gA) are shown in Fig. 14.6b. The analog ($Phe^{13,15}$gA) containing the inner pair of tryptophans (Trp-9 and -11) displays an emission maximum of 331 nm, while the analog ($Phe^{9,11}$gA) containing the outer pair of tryptophans (Trp-13 and -15) displays a red shifted emission maximum of 338 nm (the emission maximum of gramicidin in the channel conformation is 333 nm, whereas the nonchannel conformation exhibits an emission maximum of 335 nm, when excited at 280 nm) [7]. The difference in emission maximum among the analogs is indicative of differential localization of the tryptophans along the bilayer normal. Figure 14.6c shows REES data for the double tryptophan analogs. In case of the analog $Phe^{9,11}$gA, containing the outer pair of tryptophans (Trp-13 and -15), we obtained a REES of 9 nm (shift in emission maximum from 338 to 347 nm when excitation wavelength was changed from 280 to 310 nm). In contrast, the emission maximum for the analog $Phe^{13,15}$gA containing the inner pair of tryptophans (Trp-9 and -11), exhibited a shift from 331 to 349 nm as the excitation wavelength was changed from 280 to 310 nm, giving rise to an enhanced REES of 18 nm. Such large magnitudes of REES are indicative of ground state conformational heterogeneity. Further analysis employing circular dichroism and time-resolved anisotropy decay measurements established that these double tryptophan gramicidin analogs adopt different

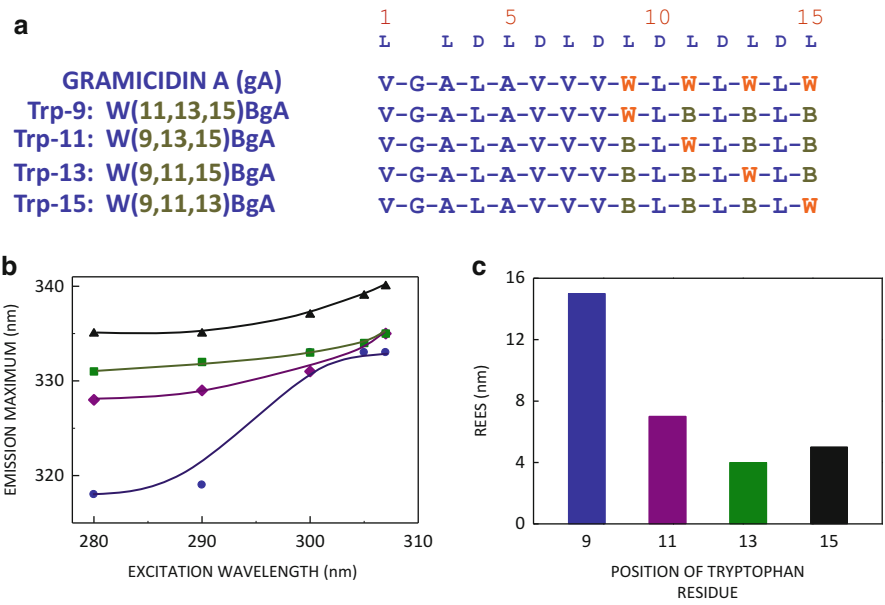

Fig. 14.5 Exploring the organization of single tryptophan analogs of gramicidin. (**a**) The positions of tryptophan and Ser-*t*-butyl (designated as B) in the amino acid sequence of gramicidin A and the single tryptophan analogs are highlighted, with alternating L- and D-residues indicated on the top. (**b**) Effect of changing excitation wavelength on the emission maximum for W(11,13,15) BgA (abbreviated as Trp-9) (●), W(9,13,15)BgA (Trp-11) (♦), W(9,11,15)BgA (Trp-13) (■), and W(9,11,13)BgA (Trp-15) (▲) in membranes. The lines joining data points are provided merely as viewing guides. (**c**) The magnitude of REES obtained for the single tryptophan analogs of gramicidin is sensitive to tryptophan depths from the centre of the bilayer. The magnitude of REES corresponds to the total shift in emission maximum when the excitation wavelength is changed from 280 to 307 nm (data shown in (**b**)) (Adapted and modified from ref. [24] with permission from Elsevier)

conformations in membranes, indicating that the conformational preference of double tryptophan gramicidin analogs is dictated by the positions of the tryptophans in the sequence. These results assume relevance in the context of the report that the inner pair of tryptophans (Trp-9 and -11) is more important for gramicidin channel formation and channel conductance [45].

In yet another study, we tested the importance of indole hydrogen bonding in gramicidin channels, by monitoring the effect of N-methylation of gramicidin tryptophans, using a combination of steady state and time-resolved fluorescence approaches [26]. As stated above, gramicidins with Trp → Phe or Tyr substitutions have greater difficulty in forming membrane-spanning dimeric channels [39, 43]. However, these results do not provide information on specific properties of tryptophan that contribute to the loss of channel structure and function. The loss in structure and function upon substitution of tryptophan with phenylalanine or tyrosine could be attributed to loss of dipole moment, hydrogen bonding ability, change in hydrophobicity, or a combination of these factors. In order to assess the

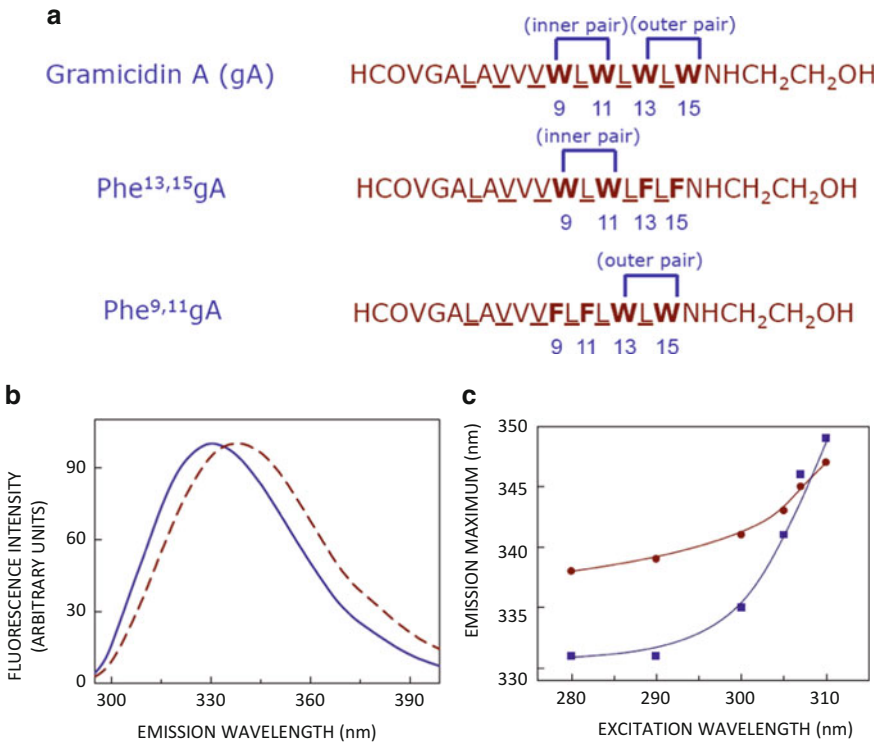

Fig. 14.6 Effect of pair-wise substitution of tryptophans in gramicidin. (**a**) To delineate the role of multiple tryptophans in the organization and dynamics of the gramicidin channel, the positions of tryptophans (four in number) in gramicidin A and the pair-wise substituted double tryptophan analogs (two in number) are shown. Tryptophan residues at positions 9 and 11 are denoted as the "inner pair", while tryptophan residues at positions 13 and 15 are denoted as the "outer pair". The analog in which the inner pair of tryptophans are substituted by phenylalanine is denoted as $Phe^{9,11}gA$, while the analog in which the outer pair of tryptophans are substituted is termed $Phe^{13,15}gA$. (**b**) Intensity-normalized fluorescence emission spectra of the two pair-wise substituted double tryptophan analogs, $Phe^{13,15}gA$ (——) and $Phe^{9,11}gA$ (---) in membranes. (**c**) Effect of changing excitation wavelength on the wavelength of maximum emission for $Phe^{13,15}gA$ (■) and $Phe^{9,11}gA$ (●). The magnitude of REES corresponds to 18 and 9 nm for the inner ($Phe^{13,15}gA$) and the outer ($Phe^{9,11}gA$) pairs, respectively. The lines joining data points are provided merely as viewing guides (Adapted with permission from ref. [25] (copyright (2014) American Chemical Society))

contribution of hydrogen bonding ability of tryptophans in maintaining the channel conformation of gramicidin, tryptophan residues were modified to 1-methyltryptophanm (see Fig. 14.7a for chemical structures of indole and 1-methylindole). This modification leads to loss of hydrogen bonding ability of tryptophans and yet, properties such as aromaticity and ring shape remain invariant. More importantly, the magnitude (~2.1 D for tryptophan and 2.2 D for 1-methyltryptophan) and direction of the dipole moment are not altered (see Fig. 14.7a) [46]. We therefore explored the membrane organization and dynamics

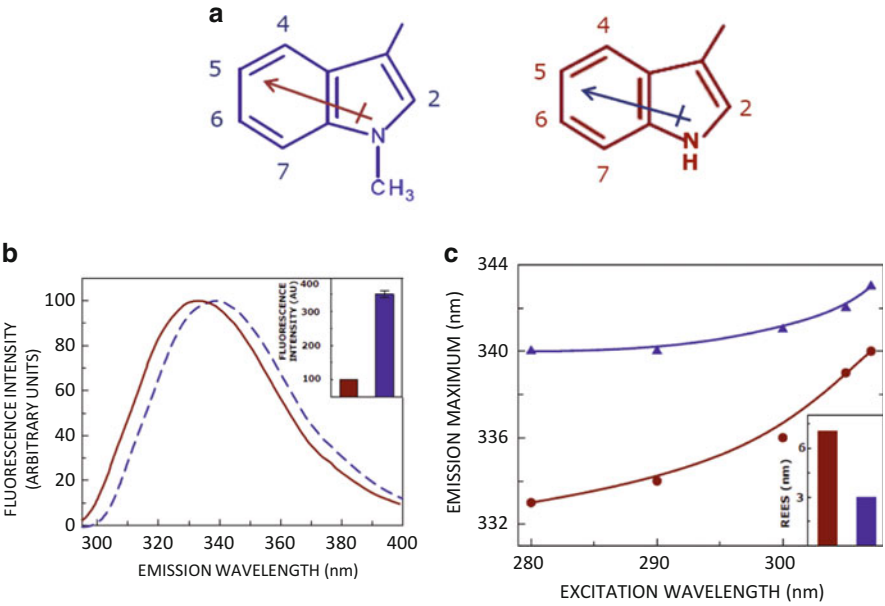

Fig. 14.7 Monitoring the effect of hydrogen bonding of the indole group of tryptophan in gramicidin. (**a**) Chemical structures of indole (*right*) and 1-methylindole (*left*) with the direction of the dipole moments are shown. The -NH group in indole can form hydrogen bond with the lipid carbonyl groups or the interfacial water molecules, while in 1-methylindole this ability is lost due the substitution with a methyl group. Importantly, aromaticity and ring shape are maintained in 1-methylindole and the dipole moment (shown as a vector) is similar in direction and magnitude (~2 D) to indole. The analog in which all four tryptophans are replaced by 1-methyltryptophan is designated as tetramethyltryptophan gramicidin (TM-gramicidin). (**b**) The intensity-normalized fluorescence emission spectra of gramicidin (——), and TM-gramicidin (- - -) in membranes are shown. The inset shows that the fluorescence intensity of TM-gramicidin (*right bar*) is higher relative to gramicidin at their respective emission maximum. (**c**) Effect of changing excitation wavelength on the wavelength of maximum emission for gramicidin (●), and TM-gramicidin (▲) in membranes. The lines joining data points are provided merely as viewing guides. The inset shows the magnitude of REES, which corresponds to the shift in emission maximum when the excitation wavelength was changed from 280 to 307 nm, is higher for gramicidin (*left bar*) (Adapted from ref. [26] with permission from Elsevier)

of the N-methylated tryptophan analog of gramicidin, *i.e.*, tetramethyltryptophan gramicidin (TM-gramicidin), a tryptophan analog of gramicidin in which all four tryptophans are replaced by 1-methyltryptophan residues. The normalized fluorescence emission spectra of gramicidin and TM-gramicidin are shown in Fig. 14.7b. The figure shows that while tryptophans in the channel form of gramicidin typically exhibit an emission maximum of 333 nm, the emission maximum of TM-gramicidin is significantly red shifted to 340 nm. The red-shifted emission maximum of TM-gramicidin is indicative of the average environment experienced by 1-methyltryptophans in TM-gramicidin due to conformational differences of gramicidin and TM-gramicidin. Interestingly, red-shifted emission maximum is

characteristic of the nonchannel conformation of gramicidin [7]. The inset in Fig. 14.7b shows that TM-gramicidin displays considerable increase in fluorescence intensity relative to gramicidin when excited at 280 nm. This could be due to the nonchannel conformation adopted by TM-gramicidin (independently shown by CD measurements) [26], since the conformational change of gramicidin in membranes from the nonchannel to channel form is accompanied by a reduction in fluorescence intensity [7]. The nonchannel conformation is characterized by increased fluorescence due to the relatively nonpolar environment in which the tryptophans are localized in the nonchannel conformation and the release of quenching due to absence of aromatic-aromatic interaction between the fluorophores at positions 9 and 15 observed in the channel conformation [25, 26]. Higher fluorescence quantum yield of 1-methyltryptophan could also contribute to increased fluorescence of TM-gramicidin. Figure 14.7c shows REES of gramicidin and TM-gramicidin. The figure shows that the emission maximum of gramicidin is characteristically shifted from 333 to 340 nm in response to a change in excitation wavelength from 280 to 307 nm, amounting to REES of 7 nm. In contrast, TM-gramicidin, exhibits a relatively modest REES of 3 nm (emission maximum shift from 340 to 343 nm) upon change in excitation wavelength from 280 to 307 nm, reminiscent of nonchannel conformation (see Fig. 14.3b) [7]. These results clearly show the importance of tryptophan hydrogen bonding in maintaining the channel conformation of gramicidin in particular and ion channels in general. In addition, these results offer the possibility that fluorescence of 1-methyltryptophan could be effectively used as a tool to explore the hydrogen bonding ability of tryptophans in membrane proteins and peptides.

14.6 Conclusions and Future Perspectives

In this review, we have focused on the application of fluorescence-based approaches to gain insight into conformational plasticity of the ion channel peptide gramicidin. Since gramicidin shares common structural motifs with more complex ion channels, the results described in this review could be useful to study conformations of more complex ion channels. This review is not meant to be an exhaustive in nature. Rather, we have provided representative applications to illustrate a specific approach that would provide novel conformational insight. In case of multitryptophan proteins, analysis of fluorescence data could be complicated due to the complexity of fluorescence processes in such systems and lack of specific information. Site-specific incorporation of extrinsic fluorescent probes, accomplished by using unnatural amino acid mutagenesis [47, 48], could help avoid this complication.

It should be mentioned here that although we have focused mainly on ion channel peptide in this review, these fluorescence-based approaches are applicable to all membrane proteins. A particularly attractive application would be to monitor conformational plasticity of G protein-coupled receptors (GPCRs) using

fluorescence-based approaches. GPCRs are involved in signal transduction from outside the cell to the cellular interior and constitute the largest family of current therapeutic targets [49]. GPCRs display remarkable structural plasticity, necessary for the functional diversity exhibited by them [50]. Unraveling conformational choices of GPCRs using fluorescence-based approaches therefore would be useful in deciphering GPCR function.

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