Monitoring ion channel conformations in membranes utilizing a novel dual fluorescence quenching approach

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Abstract

The linear peptide gramicidin forms prototypical ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics, and function of membrane-spanning channels. We have analyzed the localization of the functionally important tryptophan residues of the membrane-bound channel and non-channel conformations of gramicidin utilizing a novel dual fluorescence quenching approach [G.A. Caputo, E. London, Biochemistry 42 (2003) 3265–3274]. In this paper, we show for the first time that the dual quenching approach is applicable to multiple tryptophan containing functional ion channel peptides such as gramicidin. Importantly, dual quenching is found to be sensitive to the membrane-bound conformations of this important model ion channel.

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Ion channels are transmembrane proteins that regulate ionic permeability in cell membranes. They are crucial for normal functioning of cells and defective ion channels are implicated in a number of diseases collectively known as ‘channelopathies’ [1]. The recent successes in crystallographic analyses of ion channels starting with the KcsA potassium channel [2] have provided exciting molecular insights into ion channel structure and function. However, it is becoming increasingly clear that static crystallographic structures of membrane proteins may not always provide accurate representations of channel function [3]. Due to this ambiguity in the structural analysis of ion channel function, simple models of ion channels continue to provide useful information to understand and characterize more complex systems [4,5]. The linear peptide gramicidin forms prototypical ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics, and function of membrane-spanning channels [6,7]. Gramicidin serves as an excellent model for transmembrane channels due to its small size, ready availability, and the relative ease with which chemical modifications can be performed.

The unique sequence of alternating l- and d-chirality renders gramicidin sensitive to the environment in which it is placed [5]. Gramicidin therefore adopts a wide range of environment-dependent conformations. Two major folding motifs have been identified for gramicidin in various media: (i) the single stranded helical dimer (‘channel’ form) and (ii) the double stranded intertwined helix (collectively known as ‘non-channel’ form) [8]. Interestingly, the initial conformation adopted by gramicidin in membranes has been reported to be influenced by the nature of the solvent in which it was dissolved prior to incorporation, i.e., gramicidin conformation in membranes depends on its ‘solvent history’ [9]. However, the single stranded helical dimer conformation is the thermodynamically preferred conformation in membrane and membrane-mimetic environments [9–12].

The cation conducting gramicidin channel in membranes is formed by the head-to-head (amino terminal-to-amino terminal) dimerization of the peptide. Dual fluorescence quenching provides a method to distinguish between these two major folding motifs, and we show for the first time that this approach is applicable to gramicidin. Our results indicate that dual quenching is sensitive to the membrane-bound conformations of this important model ion channel.
terminal) single stranded β-helical dimer [13]. In this conformation, the carboxy terminus is exposed to the membrane–water interface and the amino terminus is buried in the hydrophobic core of the membrane. This places the carboxy terminal tryptophan residues clustered at the membrane–water interface at the entrance to the channel [13–16]. This interfacial localization of the gramicidin tryptophan residues is an essential aspect of gramicidin conformation and function in membranes [7]. The membrane interface seeking properties of tryptophan [17] and the oriented dipole moments of the tryptophan side chains play an important role in gramicidin conformation and ion channel activity [18–21]. While the channel conformation in membranes has been extensively investigated [14], the dynamics of the non-channel conformation is relatively unexplored [16]. Importantly, the membrane interfacial localization of tryptophan residues is absent in ‘non-channel’ conformations and the tryptophan residues are distributed along the membrane axis [16]. Such conformations have been shown to exist in membranes with polyunsaturated lipids [22] and in membranes with increased acyl chain lengths [23,24]. We have earlier utilized wavelength-selective and other sensitive fluorescence approaches to monitor the organization and dynamics of the functionally important tryptophan residues of gramicidin in the channel and non-channel conformations [16].

In this report, we have utilized a novel dual fluorescence quenching approach [25] to analyze the distribution and depths of the tryptophan residues of gramicidin in the channel and non-channel conformations. This method is based on the differing accessibility of an aqueous and membrane-bound quencher to membrane-bound tryptophan residues. The ratio of quenching (Q-ratio) by these quenchers has been shown to be related to the average depth of the fluorophore in the membrane [25]. The advantage of this approach lies in the fact that even though information on depth may be obtained by the use of a single quencher, the use of two quenchers in the Q-ratio increases sensitivity by canceling out any non-depth related effects on quenching. This work represents the first report of the dual quenching analysis of a functional multityrptophan ion channel peptide.

Materials and methods

Materials. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Gramicidin A’ (from Bacillus brevis) and 1,2-dimyrystoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Ultra pure grade acrylamide was from Invitrogen Life Technologies (Carlsbad, CA). 10-Doxynonadecane (10-DN) was a generous gift from Prof. Erwin London (SUNY, Stony Brook). The purity of acrylamide was determined along the membrane axis [16]. Such conformations and in membranes with increased acyl chain lengths [22] and in membranes with increased acyl chain lengths [23,24]. We have earlier utilized wavelength-selective and other sensitive fluorescence approaches to monitor the organization and dynamics of the functionally important tryptophan residues of gramicidin in the channel and non-channel conformations [16].

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Circular dichroism measurements. CD measurements were carried out at room temperature (25 °C) on a JASCO J-715 spectropolarimeter as described earlier [16]. Data are represented as mean residue ellipticities and were calculated using the formula:

$$[\theta] = \theta_{\text{obs}}/(10C),$$

where $\theta_{\text{obs}}$ is the observed ellipticity in mdeg, $l$ is the path length in cm, and $C$ is the concentration of peptide bonds in mol/L.

Results and discussion

The initial conformation that gramicidin adopts when incorporated into membranes is dependent on the nature of the solvent in which it was dissolved prior to incorporation in membranes [9,10]. Thus, when gramicidin is dissolved in solvents such as chloroform/methanol or ethanol before incorporation into membranes, it tends to adopt double helical non-channel conformations. Upon sonication and incubation at 65 °C, such conformations are converted to the characteristic channel conformation. We used the ethanol injection method [30] to generate non-channel conformations of gramicidin in POPC vesicles. Gramicidin incorporated in membrane vesicles this way has been shown to initially adopt the non-channel conformation [16]. Fig. 1A shows representative circular dichroism spectra for the channel and non-channel conformations obtained this way. The intensity-normalized fluorescence emission spectra of the channel and non-channel conformations of gramicidin are shown in Fig. 1B. When excited at 280 nm, gramicidin tryptophans in the channel form exhibit an emission maximum of 333 nm. The emission maximum of the non-channel form, on the other hand, displays a slight red shift and is at 335 nm, in agreement with previous literature [16,31]. In addition, the fluorescence intensity of the non-channel form is increased compared to the channel form (not shown). This is clearly indicative of differing average environments for the gramicidin tryptophans in the channel and non-channel conformations. Previous work from our laboratory utilizing wavelength-selective fluorescence indicated that the gramicidin tryptophans in the channel conformation are localized at the membrane interfacial region characterized by restricted motional reorientation [15,16]. In contrast, the average environment of tryptophans in the non-channel form is considerably less restricted as evidenced by the reduced magnitude of red edge excitation shift (REES) observed in this conformation [16].

Quenching of tryptophan fluorescence using aqueous quenchers such as acrylamide is a widely used tool to monitor tryptophan environments in proteins [32]. Interestingly, acrylamide quenching of gramicidin tryptophan fluorescence was found to be insensitive to the conformation of membrane-bound gramicidin as evaluated by the Stern–Volmer constant ($K_{SV}$) and the bimolecular quenching constant ($k_q$) [16]. Thus, while $K_{SV}$ values indicated an increased accessibility to the aqueous environment for the non-channel tryptophans, the values for bimolecular quenching constants however did not support this interpretation. This is in contrast to other sensitive fluorescence parameters such as REES which indicated a deeper average location for gramicidin tryptophans in the non-channel conformation [16]. In addition, chemical modification of tryptophan residues using N-bromosuccinimide (NBS) as an oxidant also did not provide a clear picture for the distribution of tryptophan residues in these conformations [16].

In the present work, we have addressed the issue of relative depths of gramicidin tryptophans in the channel and
non-channel conformations by using a novel dual quenching approach [25] which eliminates some of the complications associated with earlier measurements. This method utilizes two quenchers, the aqueous quencher acrylamide and membrane-bound spin label quencher 10-doxyl-nondecane (10-DN), to calculate a quenching ratio (Q-ratio) which has been found to have an approximate linear relationship with fluorophore depth in the membrane [25]. Even though information on depth may be obtained by the use of a single quencher (such as acrylamide), the use of two quenchers in the Q-ratio amplifies sensitivity by canceling out any non-depth related effects on quenching. 10-DN is a derivative of the aliphatic hydrocarbon nonadecane and contains a nitroxide-bearing doxyl group (spin label). Spin labels are known to be strong quenchers of a wide range of fluorophores including tryptophans [33–36]. 10-DN is a hydrophobic molecule and does not have a polar moiety to anchor the nitroxide group in the membrane at a fixed depth, in contrast to spin-labeled phosphatidylecholines, where the spin label is known to be at a very specific location (depth) in the membrane bilayer [34,35].

Fig. 2 shows the quenchings of gramicidin tryptophan fluorescence obtained in the presence of fixed concentrations of the aqueous quencher acrylamide and the membrane-bound quencher 10-DN. As discussed above, acrylamide quenching does not exhibit appreciable sensitivity to the different conformations of gramicidin. Importantly, fluorescence quenching by 10-DN appears to be more sensitive to the channel and non-channel conformations of gramicidin. The Q-ratios calculated according to Eq. (2) are shown in Fig. 2. The Q-ratio in the non-channel conformation (0.27) is low while the Q-ratio for the channel conformation (0.69) is relatively high. The lower Q-ratio for the non-channel conformation is consistent with tryptophan residues embedded in the deeper regions of the membrane in this conformation, while a higher Q-ratio for the channel conformation is consistent with tryptophans embedded in the deeper regions of the membrane bilayer. However, the dual quenching approach has some advantages over more quantitative approaches such as parallax [34] and distribution [37].

![Fig. 2. Quenching and Q-ratios of gramicidin fluorescence in the non-channel and channel conformations.](image)

![Fig. 3. Schematic representation of the channel and non-channel conformations of gramicidin indicating the location of tryptophan residues in a membrane bilayer.](image)
analysis. In addition, since this approach utilizes quenchers at very different locations (aqueous and membrane-bound), the difference in quenching is large and more easily measured when compared to other methods where the quenchers (spin-labeled or brominated lipids) are placed at only slightly different depths.

The \(Q\)-ratio was originally calibrated and found to be linear for \(\alpha\)-helical peptides with a single tryptophan placed at different positions in the sequence of the transmembrane helix [25]. However, the \(Q\)-ratios obtained by us for gramicidin in the channel and non-channel conformation are for a multitransport peptide. Nonetheless, the overall agreement of the \(Q\)-ratios with the relative location of tryptophan residues in the membrane bilayer even for a multitransport peptide such as gramicidin is indeed encouraging. It would be interesting to compare \(Q\)-ratios of analogues of gramicidin containing single tryptophan residues [18,38] at defined locations in the membrane bilayer.

Importantly, 10-DN does not anchor at any specific location and can therefore be easily accommodated in membranes of different hydrophobic thicknesses [25]. This is particularly useful to study the organization and conformation of tryptophan containing peptides in cases of hydrophobic mismatch [25,39–41]. \(Q\)-ratios therefore provide a relative scale for depth of the fluorophore in the membrane that can be conveniently used across different membrane systems. Dual quenching analysis has been found to be useful to analyze the membrane penetration of membrane peptides, utilizing both intrinsic tryptophan fluorescence and external fluorophores [41–43]. This report shows, for the first time, that the dual quenching analysis could be applicable to functional multitransport peptides such as gramicidin. Such an approach could prove especially useful to analyze the membrane thickness induced conformational response of such multiple tryptophan peptides.

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