

Monitoring Membrane Protein Conformational Heterogeneity by Fluorescence Lifetime Distribution Analysis Using the Maximum Entropy Method

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Abstract Due to the inherent difficulty in crystallizing membrane proteins, approaches based on fluorescence spectroscopy have proved useful in elucidating their conformational characteristics. The ion channel peptide gramicidin serves as an excellent prototype for monitoring membrane protein conformation and dynamics due to a number of reasons. We have analyzed conformational heterogeneity in membrane-bound gramicidin using fluorescence lifetime distribution analysis of tryptophan residues by the maximum entropy method (MEM). MEM represents a model-free and robust approach for analyzing fluorescence lifetime distribution. In this paper, we show for the first time, that fluorescence lifetime distribution analysis using MEM could be a convenient approach to monitor conformational heterogeneity in membrane-bound gramicidin in particular and membrane proteins in general. Lifetime distribution analysis by MEM therefore provides a novel window to monitor conformational transitions in membrane proteins.

Keywords Membrane proteins · Gramicidin · Ion channel · Fluorescence lifetime distribution · Maximum entropy method

Introduction

About 30% of all open reading frames (ORFs) are predicted to encode membrane proteins and almost 50% of all proteins encoded by an eukaryotic genome are membrane proteins [1, 2]. Integral membrane proteins play crucial roles in various cellular processes. However, membrane proteins are difficult to crystallize in their native conditions due to the intrinsic dependence on surrounding membrane lipids [3]. It is against this backdrop that approaches based on fluorescence spectroscopy have proved useful in elucidating the organization, topology and orientation of membrane proteins and peptides [4].

The linear peptide gramicidin forms ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics, and function of membrane-spanning channels [5]. Gramicidin represents an excellent prototype for monitoring membrane protein conformation and dynamics due to its small size, ready availability and the relative ease with which chemical modifications can be performed [6]. The unique sequence of alternating L- and D-chirality renders gramicidin sensitive to the environment in which it is placed. Gramicidin therefore adopts a wide range of environment-dependent conformations. In spite of the alternating sequence of L-D chirality generally not encountered in naturally occurring peptides and proteins, gramicidin represents a useful model for realistic determination of conformational preference of proteins in a membrane environment. This is due to the fact that the dihedral angle combinations generated in the conformation space by various gramicidin conformations are allowed according to the Ramachandran plot [7].

Gramicidin has been shown to assume two major folding motifs in various media: (i) the single stranded helical dimer ('channel' form), and (ii) the double stranded

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intertwined helix (collectively known as ‘non-channel’ form) (see Fig. 1) [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) single stranded $\beta^{6.3}$ 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 conformation 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 [5, 17]. The membrane interface seeking properties of tryptophan [18] and the oriented dipole moments of the tryptophan side chains play an important role in gramicidin conformation and ion channel activity [19–22]. Interestingly, the membrane interfacial localization of tryptophan residues is absent in ‘non-channel’ conformations and the tryptophan residues are distributed along the membrane axis (see Fig. 1) [16]. Such non-channel conformations have been shown to exist in membranes with polyunsaturated lipids [23], and in membranes with increased acyl chain lengths under hydrophobic mismatch conditions [24, 25].

In this report, we have monitored conformational heterogeneity in membrane-bound gramicidin using fluorescence lifetime distribution analysis of tryptophan residues by the maximum entropy method (MEM). MEM represents a model-free approach for analyzing fluorescence lifetime distribution [26–28]. The width of the lifetime distribution obtained by this method is correlated with the degree of heterogeneity of the environment as sensed by the fluorophore [29, 30]. Our results represent one of the early reports on the application of fluorescence lifetime distribution analysis using MEM to explore conformational heterogeneity in membrane proteins.

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-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, MO). The concentration of gramicidin was calculated from its molar extinction coefficient (ϵ) of $20,700 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm [10].

Sample preparation

All experiments were done using unilamellar vesicles (ULV) of POPC containing 2% (mol/mol) gramicidin A'. The channel and non-channel conformations of gramicidin were generated essentially as described earlier [16]. All experiments were carried out at $\sim 23 \text{ }^\circ\text{C}$.

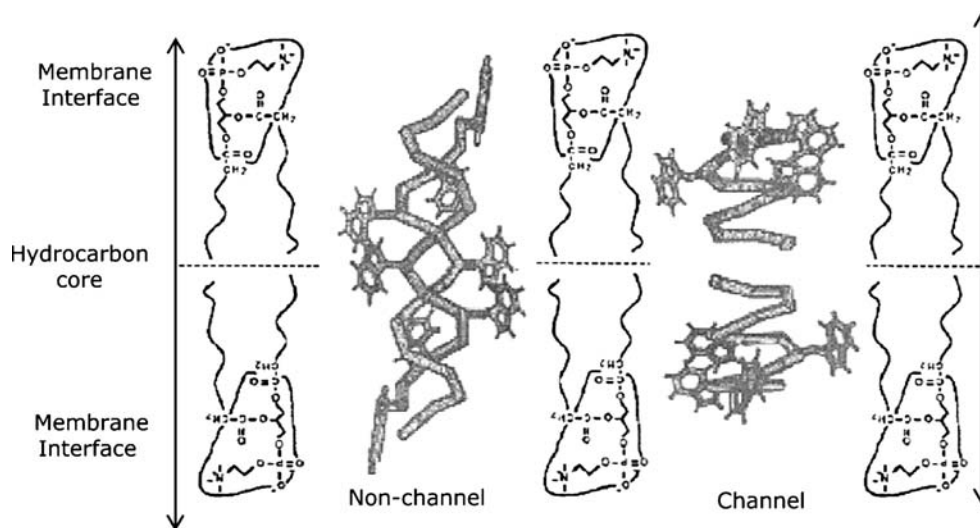


Fig. 1 A schematic representation of the channel and non-channel conformations of gramicidin indicating the location of tryptophan residues in a membrane bilayer. The tryptophan residues are clustered toward the membrane interface in the channel conformation, whereas

they are distributed along the membrane axis in the non-channel conformation. The membrane axis is represented by double headed arrows and the center of the bilayer is shown as a dotted line. See text for details (adapted and modified from [16])

Steady state fluorescence measurements

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used. Background intensities of samples in which gramicidin was omitted were negligible in most cases and were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts.

Time-resolved fluorescence measurements

Time-resolved fluorescence intensity decay measurements were carried out using a time correlated single photon counting (TCSPC) setup. For fluorescence lifetime measurements, 1 ps pulses of 887 nm radiation from the Ti-sapphire femto/pico second laser (Spectra Physics, Mountain View, CA), pumped by an Nd-YLF laser (Millenia X, Spectra Physics), were frequency tripled to 295 nm by using a frequency doubler/tripler (GWU, Spectra physics). Fluorescence decay curves were obtained at the laser repetition rate of 4 MHz by a micro-channel plate photomultiplier (model R2809u, Hamamatsu Corp.) coupled to a TCSPC setup. The instrument response function (IRF) at 295 nm was obtained using a dilute colloidal suspension of dried non-dairy creamer. The full width at half maxima (FWHM) of the IRF was 40 ps and number of channels used was 1,024. Fluorescence emission measurements of gramicidin, excited at 295 nm, were carried out at 340 nm, using a combination of a monochromator and a 320 nm cut-off filter. Fluorescence intensity decay was collected from the sample after excitation with the emission polarizer oriented at the magic angle (54.7°) with respect to the excitation polarizer. The fluorescence emission at magic angle (54.7°) was dispersed in a monochromator (spectral width 2.5 nm) and counted ($3\text{--}4 \times 10^3 \text{ s}^{-1}$) by a microchannel plate photomultiplier, and processed through constant fraction discriminator, time-to-amplitude converter and multichannel analyzer. To optimize the signal to noise ratio, 20,000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits with a nominal bandpass of 3 nm or less. The data stored in the multichannel analyzer was routinely transferred to an IBM PC for analysis. Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a preexponential factor representing the fractional contribution

to the time-resolved decay of the component with a lifetime τ_i such that $\sum_i \alpha_i = 1$. The decay parameters were recovered using a nonlinear least squares iterative fitting procedure based on the Levenberg-Marquardt algorithm [31, 32]. A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.2. The fluorescence decays were analyzed by the discrete exponential analysis as well as the maximum entropy method (MEM). Amplitude-averaged lifetimes (τ_m) and intensity-averaged lifetimes ($\langle \tau \rangle$) for triexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equations [33, 34]:

$$\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3 \quad (2)$$

$$\langle \tau \rangle = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2 + \alpha_3 \tau_3^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3} \quad (3)$$

Circular dichroism (CD) measurements

CD measurements were carried out at room temperature ($\sim 23^\circ \text{C}$) on a JASCO J-815 spectropolarimeter as described earlier [16].

Results and discussion

The natural mixture of gramicidins, often denoted as gramicidin A' (gramicidin D in older literature), consists of $\sim 85\%$ of gramicidin A, which has four tryptophan residues at positions 9, 11, 13 and 15. Gramicidin A' is readily available commercially and is fluorescent, due to the presence of the tryptophan residues. Tryptophan residues in gramicidin channels are believed to be crucial for maintaining the structure and function of the channel [6]. 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]. For example, 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 [35] to generate non-channel conformations of gramicidin in POPC vesicles. Gramicidin incorporated in membrane vesicles this way has been shown to adopt the non-channel conformation [16].

Figure 2a shows representative circular dichroism spectra for the channel and non-channel conformations

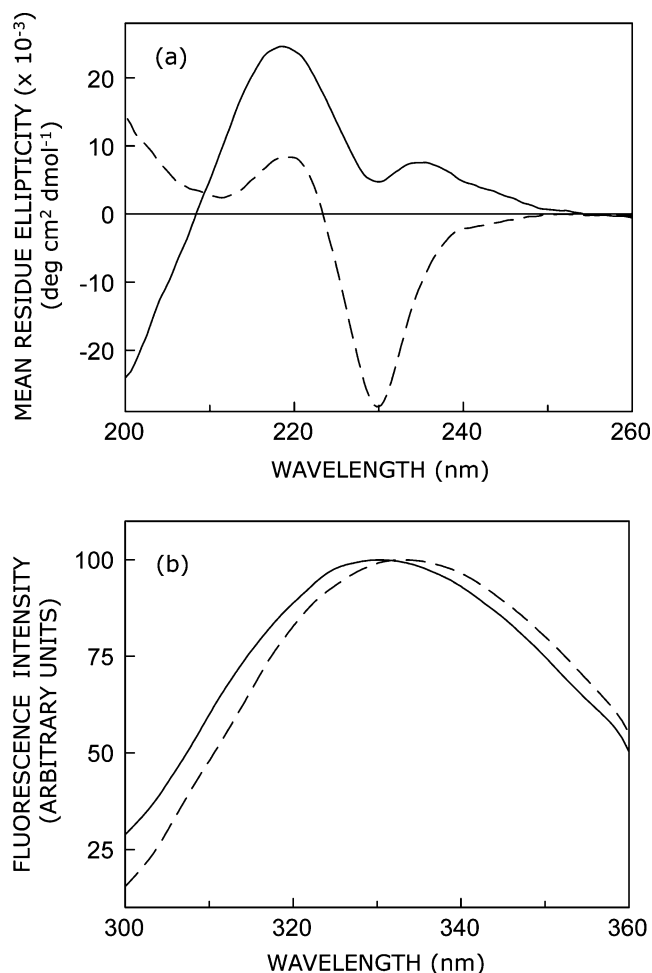


Fig. 2 **a** Far UV CD spectra of the channel (—) and non-channel (---) forms of gramicidin in vesicles of POPC. **b** Fluorescence emission spectra of the channel (—) and non-channel (---) forms of gramicidin in vesicles of POPC. The excitation wavelength was 280 nm. The spectra are intensity-normalized at the emission maximum. The two forms of gramicidin were generated as described in [Materials and methods](#). The ratio of gramicidin to POPC was 1:50 (mol/mol) and the concentration of POPC was 0.85 mM. See [Materials and methods](#) for other details

obtained this way. The intensity-normalized fluorescence emission spectra of the channel and non-channel conformations of gramicidin are shown in [Fig. 2b](#). 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](#), [36](#)]. This is indicative of different average environments for the gramicidin tryptophans in the channel and non-channel conformations. Previous work from our laboratory, utilizing the wavelength-selective fluorescence approach [[37](#)], showed that the gramicidin tryptophans in the channel conformation are localized at the membrane interfacial region characterized by motional restriction [[15](#), [16](#)]. On the other hand, 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](#)].

Fluorescence lifetime serves as a sensitive indicator of the local environment and polarity in which a given fluorophore is placed [[38](#)]. [Table 1](#) shows gramicidin tryptophan lifetimes analyzed by the discrete analysis method. As can be seen from the table, both fluorescence decays could be fitted well with a triexponential function. The mean amplitude-averaged lifetimes (τ_m) and intensity-averaged lifetimes ($\langle\tau\rangle$) of gramicidin tryptophans were calculated using [Eqs. 2](#) and [3](#) and are shown in [Table 1](#). The mean fluorescence lifetime for gramicidin tryptophans are found to be longer in the non-channel form than the channel form, irrespective of the method of calculation. In general, tryptophan lifetimes are known to be reduced when exposed to polar environments [[39](#)]. The reduction in mean fluorescence lifetime of the tryptophans in the channel conformation can be attributed to the polarity of the interfacial region where the channel tryptophans are localized. Another possible reason for the higher fluorescence lifetime in the non-channel form could be the release of stacking interaction between the indole rings of Trp-9 and 15. These tryptophan residues have been previously implicated in specific aromatic–aromatic (stacking) interaction in the $\beta^{6.3}$ helical channel form [[15](#)].

Fluorescence decay kinetics of fluorophores in complex systems generally display a considerable level of heterogeneity. The lifetime distribution of fluorophores in such cases represents a powerful method for characterizing complex heterogeneous systems such as membranes. The width of the lifetime distribution has been used to interpret the integrity and heterogeneity of such systems [[29](#), [30](#)]. Lifetime distribution analysis by MEM represents a convenient, robust and model-free approach of data analysis [[26–28](#)]. In MEM, the fluorescence intensity decay ($I(t)$) is analyzed using the model of continuous distribution of lifetimes:

$$I(t) = \int_0^{\infty} \alpha(\tau) \exp(-t/\tau) d\tau \quad (4)$$

Here, $\alpha(\tau)$ represents the amplitude corresponding to the lifetime τ in the intensity decay. In practice, the limits on the above integration are set based on the information regarding the system under study and the detection limit of the instrument. In our case, the lower and the upper limits of the integration was set as 10 ps and 10 ns, respectively. For practical purposes, the above equation can be written in terms of a discrete sum of exponentials as

$$I(t) = \sum_{i=1}^N \alpha_i \exp(-t/\tau_i) \quad (5)$$

Table 1 Representative fluorescence lifetimes of gramicidin conformations^a

Gramicidin conformation	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	τ_m^b (ns)	$\langle\tau\rangle^c$
Channel	0.10	3.81	0.50	1.39	0.40	0.43	1.24	1.98
Non-channel	0.16	4.84	0.40	1.66	0.44	0.50	1.66	2.99

^a The excitation wavelength was 295 nm and emission was monitored at 340 nm. All other conditions are as in Fig. 2. The errors were 5–10% for all parameters. See [Materials and methods](#) for other details

^b Calculated using Eq. 2

^c Calculated using Eq. 3

where N represents the total number of exponentials. In our data analysis, N is taken as 100 exponentials equally spaced in the $\log(\tau)$ space between the lower and upper limits. MEM initially starts with a flat distribution of amplitudes $\alpha(\tau)$, i.e., each lifetime has equal contribution in the beginning and arrives at the amplitude distribution which best describes the observed experimental fluorescence intensity decay. The optimization of the amplitude distribution $\alpha(\tau)$ is carried out in successive cycles by minimizing the χ^2 value (0.96 in both cases) and maximizing the entropy (S) [26]. The expression used for S is the Shannon-Jayne's entropy function, which is

$$S = -\sum p_i \log p_i \quad \text{where } p_i = \alpha_i / \sum \alpha_i \quad (6)$$

Successive iterations provide distribution that minimize χ^2 and maximize S . If the χ^2 criterion is satisfied by many distributions in a particular iteration, then the distribution with maximum entropy is selected. The analysis is terminated when χ^2 reaches the specified lower limit or when χ^2 and $\alpha(\tau)$ show no change in successive iterations. MEM analysis gives a lifetime distribution that is model independent. Interestingly, although lifetime distribution using MEM has been used to study both membranes [29, 30] and proteins [27], these results constitute one of the early reports for analysis of conformational heterogeneity in membrane proteins by fluorescence lifetime distribution analysis using MEM.

Figure 3 shows the lifetime distribution obtained by MEM analysis of tryptophan residues in the channel and non-channel forms of gramicidin. Interestingly, lifetime distribution of tryptophan residues in the non-channel form ($w=3.40$ ns) was found to be significantly broader than the channel form ($w=0.96$ ns) [w denotes the width of the fluorescence lifetime distribution, represented as full width at half maxima (FWHM) for the major peak]. This shows that the tryptophans in the non-channel form experience relatively heterogeneous environment than the environment experienced in the channel form. The relatively homogeneous environment experienced by the tryptophans in the channel form is in line with the observation that structured proteins display relatively sharp distribution of tryptophan lifetimes, compared to the distributions obtained with

denatured proteins [40]. This is consistent with our previous result in which we showed that the tryptophan residues are clustered at the membrane interfacial region in the channel form, while they are spread all across the bilayer normal in case of the non-channel form (see Fig. 1)

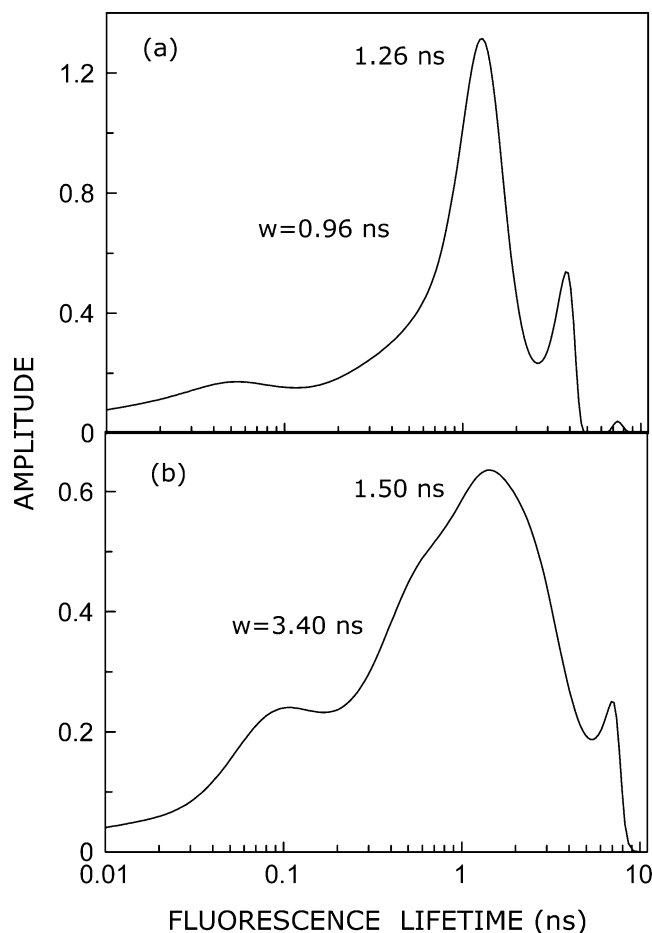


Fig. 3 MEM lifetime distribution of tryptophan residues for the **a** channel and **b** non-channel conformations of gramicidin. Here ' w ' represents the width of the fluorescence lifetime distribution (represented as full width at half maxima, FWHM) for the major peak whose positions are indicated on the representative distributions. The ratio of gramicidin to POPC was 1:50 (mol/mol) and the concentration of POPC was 0.85 mM. See [Materials and methods](#) for other details

[16]. In other words, the distributions and depths of the gramicidin tryptophans represent major differences between the two forms. Interestingly, the mean fluorescence lifetimes (shown in Table 1) and the peak positions (1.26 ns for the channel form and 1.50 ns for the non-channel form, see Fig. 3) from the lifetime distribution analysis by MEM, follow similar trend.

We show here that difference in conformation for membrane proteins can be monitored by fluorescence lifetime distribution analysis using MEM, which is sensitive to the difference in conformational heterogeneity among the two forms (Fig. 3). Although fluorescence lifetime of a membrane protein has earlier been analyzed using MEM, it was not used to address the issue of conformational heterogeneity [41]. The reason that the non-channel conformation is not the thermodynamically stable form in membranes is due to the fact that in this form some of the tryptophan residues are buried in the low dielectric nonpolar region of the membrane, which is energetically unfavorable [18]. The interfacial localization of the gramicidin tryptophan residues in the channel conformation is an essential aspect of gramicidin conformation and function in membranes. In general, tryptophan residues in membrane proteins are not uniformly distributed along the membrane axis and tend to be preferentially clustered at the membrane interface [17].

As discussed previously, gramicidin represents a useful model for realistic determination of conformational preference of proteins in a membrane environment. The conformational preferences of membrane-bound gramicidin are therefore not unique to it. We can consider the property of conformational transition of gramicidin between functional (channel) and non-functional (non-channel) states, to be analogous to the conformational changes that occur during the switch between 'on' and 'off' states during the activation of membrane-bound receptors and channels. This is exemplified by a recent example from the KirBac1.1 channel, where it was shown that localization of aromatic amino acids in the membrane interior could result in a closed conformation in case of the channel [42], analogous to what is observed in the case of gramicidin. We conclude that lifetime distribution analysis by MEM provides a novel window to monitor such conformational transitions in membrane proteins.

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