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Photoinduced Electron Transfer and Fluorophore Motion as a Probe of the Conformational Dynamics of Membrane Proteins: Application to the Influenza A M2 Proton Channel

Julie M. G. Rogers^{2,&}, Alexei L. Polishchuk^{2,&}, Lin Guo^{1,&}, Jun Wang¹, William F. DeGrado^{1,2}, and Feng Gai^{1,*}

¹Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, USA

²Department of Biochemistry and Molecular Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

The structure and function of the Influenza A M2 proton channel have been the subject of intensive investigations in recent years because of its critical role in the life cycle of the Influenza virus. Using a truncated version of the M2 proton channel (i.e., M2TM) as a model, here we show that fluctuations in the fluorescence intensity of a dye reporter that arise from both fluorescence quenching via the mechanism of photoinduced electron transfer (PET) by an adjacent tryptophan (Trp) residue and local motions of the dye molecule can be used to probe the conformational dynamics of membrane proteins. Specifically, we find that the dynamics of the conformational transition between the N-terminally-open and C-terminally-open states of the M2TM channel occur on a timescale of about 500 μ s and that binding of either amantadine or rimantadine does not inhibit the pH-induced structural equilibrium of the channel. These results are consistent with the direct occluding mechanism of inhibition which suggests that the antiviral drugs act by sterically occluding the channel pore.

Keywords

Fluorescence correlation spectroscopy; M2 proton channel; Conformational dynamics; Photo-induced electron transfer

INTRODUCTION

The M2 proton channel of the influenza A virus is composed of four relatively small (97 residues) monomers that span the viral envelope as a helical transmembrane bundle¹. Among its several functions,^{2,3} M2 mediates acidification of the viral interior from an acidic endosome lumen, which enables release of viral RNA in a form competent for replication within the host cell.¹ Thus, it has been identified as a major target for anti-influenza drugs.⁴ Recently, it has been shown that the tetrameric assembly, proton translocation function, and the inhibition of full length M2 are maintained by a remarkably small, 25-residue peptide, M2TM.³ Because of its smaller size, the M2TM transmembrane helical bundle provides a

*To whom correspondence should be addressed. gai@sas.upenn.edu.

&These authors contributed equally to this work.

Supporting Information Available. Control experiments, residual plots, and fitting parameters. This material is available free of charge via the Internet at <http://pubs.acs.org>

unique model system for examining the structural and dynamic determinants of M2 function.

While the mechanism of proton translocation through M2 has been a subject of much debate,^{5,6} two amino acids within the transmembrane domain, His37 and Trp41, have been shown to be critical to M2 proton translocation, with His 37 mediating proton selectivity and pH activation of transport,^{7,8} and Trp41 being required to facilitate largely unidirectional proton flow across the membrane in an N-terminal to C-terminal fashion.⁹ Recently, several high-resolution structures of the M2 transmembrane bundle have been obtained,¹⁰⁻¹² suggesting that the conformation of the tetrameric helical bundle changes significantly in response to a pH change, as the bundle assumes a cylinder shape at high pH (referred to as N-open hereafter), but a cone shape at low pH where the C-terminal ends of the transmembrane helices become further separated (referred to as C-open hereafter). This pH-induced conformational change along with a concerted rotamer shift of the Trp41 sidechain, which interacts with His37 in a cation- π like fashion,¹³ are believed to be key determinants of the M2 proton translocation capability.¹⁴ It has been hypothesized that the mechanism is transporter-like, involving the protonation of His37 from the N-terminal side via an open N-terminal pore while the C-terminal pore exit is closed off by Trp41 sidechains.¹⁴ After a conformational shift that closes off the N-terminal end of the bundle and opens the C-terminal end, His37 releases its proton to pore waters on the C-terminal side of the membrane.¹⁴ In this model, the opening/closing motions of the channel occur at all pHs, and a change in pH induces a shift in the equilibrium between N-open and C-open,^{14,15} and possibly other stable intermediates.

While this mechanistic hypothesis is supported by a recent molecular dynamics simulation,¹⁴ a direct experimental assessment of the underlying conformational transitions upon protonation of M2 tetramer is still lacking. In addition, as a result of two conflicting high resolution structures, the mechanism of inhibition by two extensively used anti-influenza agents, amantadine and rimantadine,¹⁶ is also under debate. One view is based on a crystal structure, in which amantadine was found in the interior of the channel, and suggests that the drug functions by occluding the channel pore.¹¹ The other view, which is based on an NMR structure in which the rimantadine binding site was outside the channel, suggests that conduction is prevented allosterically, and that drug binding stabilizes the N-open conformation.¹⁰ More recently, an NMR structure showed that amantadine is bound to the channel pore at stoichiometric concentrations, and to the outside of the channel only when the concentration is increased.¹²

To provide further insight into the pH-induced structural changes of the M2 channel embedded in fully hydrated membranes, we use fluorescence correlation spectroscopy (FCS) in conjunction with photoinduced electron transfer (PET) to measure how the fluctuation dynamics of the Trp41 sidechain in M2TM change as a function of pH. Via the mechanism of PET, Trp can effectively quench the fluorescence of a series of oxazine dye molecules, such as MR121 and Atto 655, when the indole ring and the dye of interest are brought sufficiently close to one another.^{17,18} Thus, the protein conformational dynamics involving motions that result in changes in the separation distance between Trp and the dye reporter could be probed via FCS, as such motions would induce fluctuations in the fluorescence of the reporter. Herein, we introduce an Atto 655 dye at position 40 via a Cys mutant of M2TM, Leu40/Cys (the labeled peptide is hereafter referred to as Atto-40-M2TM) and use PET to probe the M2TM channel dynamics. In addition, we show that the dynamic motion of the dye molecule is also a useful probe of the dynamics and/or conformational equilibrium of the M2TM tetramer. Taken together, our results support the transporter-like mechanism^{14,15} and also the measurements indicating that drugs act by occluding the channel.^{11,12}

EXPERIMENTAL SECTION

Peptide synthesis and purification

Peptides were synthesized by solid-phase Fmoc synthesis as C-terminal amides, cleaved and purified as previously described.¹⁹ The WT M2TM peptide (22–46), (sequence: SSDPLVVAASIIGILHLILWILDRL), the M2TM W41F variant was not N-terminally acetylated, while an N-terminal acetyl group was installed on the WT M2TM 19–46 peptide (M2TM 22–46 with an additional sequence of CND at the N-terminus) and the other Cys-containing peptides used in these experiments (M2TM L40C, M2TM L40C W41F, and M2TM I32C). Following RP-HPLC purification, peptides were lyophilized, the purity was assayed by analytical RP-HPLC and the identity was confirmed by MALDI-TOF mass spectrometry.

Peptide labeling

M2TM peptides were labeled with ATTO 655 maleimide (Atto-Tec, Siegen, Germany or Sigma-Aldrich, St. Louis, MO). Labeling reactions were performed with a ~10–20x excess of dye to peptide. Peptide concentrations were quantified by UV-Vis spectrophotometry in mixtures of acetonitrile and water that provided optimum solubility. An extinction coefficient of $5500 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm was used for Trp-containing peptides and an extinction coefficient of $195 \text{ M}^{-1}\text{cm}^{-1}$ at 257 nm was used for W41F mutants. The purchased dye solid (1 mg) was dissolved in acetonitrile. Peptide and dye stock were added to an acetonitrile-water mixture with added sodium phosphate buffer at pH ~7.3. Total reaction mixture volumes were typically less than 0.5 mL. The reaction mixture was stirred at room temperature and monitored by reverse-phase analytical HPLC, using similar gradients as preparative purification methods for M2TM peptides.¹⁹ Reactions were usually complete within 3 hours, as evidenced by the absence of the starting material peak from the trace and the appearance of a new peak, absorbing strongly at 630 nm and eluting near the time of the starting material peak. Upon completion, reaction mixtures were quenched with a drop of trifluoroacetic acid and diluted as necessary to facilitate handling. Owing to the small amount of sample, labeled peptides were purified using serial analytical HPLC injections of the reaction mixture. Labeled peptide fractions (eluting as a blue colored solution) were lyophilized, and assayed for identity and purity by MALDI-TOF mass spectrometry.

M2TM reconstitution into small unilamellar vesicles

Lipid films (2.5 μmol total lipid) of 4:1 POPC:POPG (Avanti Polar Lipids, Alabaster, AL) were prepared by co-drying chloroform stocks of the phospholipids under a stream of nitrogen or argon followed by lyophilization and storage at -20°C . 6.25 nmol of unlabeled peptide (1:400 monomer:lipid ratio) in 40 mM octylglucoside ($<50 \mu\text{L}$) was added dropwise to the lipid film, followed by 0.25 nmol (1:10,000 monomer:lipid ratio) of labeled peptide ($<10 \mu\text{L}$). The W41F variant peptides (labeled and unlabeled) were added to the lipid film as ethanolic stocks because of limited solubility in octylglucoside; the mixture was vortexed and re-dried under a stream of inert gas. Extinction coefficients for unlabeled peptides were as above, whereas labeled peptides were quantified using the dye absorption maximum of $1.25 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ near 660 nm, where the peptide components have no additional absorbance.

The sample mixture was buffered with 5 mM NaMOPS pH 7 and 100 mM NaCl, and the volume increased to 500 μL with periodic vortexing. The mixture was freeze-thawed 15 times, and then the volume was increased to 1 mL. The sample was dialyzed overnight against 1–2 volumes of 1L 5 mM NaMOPS pH 7 and 100 mM NaCl using 10 kDa cutoff Slide-A-Lyzer cassettes (Pierce, Rockford, IL), with Amberlite XAD-4 resin added to bind

residual OG. Following dialysis, the sample was sonicated extensively on ice using a Fisherbrand Model 500 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), followed by centrifugation in a benchtop microcentrifuge to sediment sonicator tip shavings and any aggregates. The supernatant was isolated and maintained on ice prior to experiments, which were performed within 4 days of sample preparation.

FCS sample preparation

For M2 experiments, liposome samples were diluted 1:10 into buffers consisting of 80 mM NaCl and 25 mM NaMOPS (higher pH ranges) or NaOAc (lower pH ranges), depending on the desired pH. Some samples, as described in the text, contained either 100 μ M amantadine or 20 μ M rimantadine. Samples were placed on glass coverslips that had been modified with 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (Gelest, Morrisville, PA). After acid washing, glass coverslips were treated with a solution which was prepared as follows: the pH of a 95% ethanol, 5% water solution was adjusted to 5.3 with acetic acid, 2% (by volume) 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane was then added and allowed to sit for 5 minutes (to allow hydrolysis). Coverslips were added to solution for 2 minutes, then removed and rinsed vigorously with ethanol and allowed to dry for 24 hours. Coverslips were further rinsed with water and dried before use.

FCS setup and data analysis

The details of the FCS setup have been described elsewhere.²⁰ In the current case, excitation of the Atto 655 dye was accomplished by the ~ 30 μ W 630 nm line of a Helium Neon laser (JDS Uniphase Corporation, Milpitas, CA). Each FCS curve was accumulated for a duration of 300 s and an average of 10 to 30 such FCS curves was used in the subsequent data analysis. The FCS data were fit to the following equation (or other models discussed in the text):²¹

$$G(\tau) = \frac{1}{N} \left(\left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \left(\frac{1}{1 + \frac{\tau}{\omega^2 \tau_D}} \right)^{1/2} \right) \times \left(\frac{1 - \sum_{i=1}^m (T_i - T_i \cdot \exp(-\frac{\tau}{\tau_i}))}{1 - \sum_{i=1}^m T_i} \right) \quad (1)$$

where τ_D represents the 3-D diffusion time constant, ω refers to the axial to lateral dimension ratio of the confocal volume element, N represents the number of fluorescent molecules in the confocal volume, τ_i and T_i represent the respective time constant and amplitude of the dynamic component i . The values of ω were determined by fitting the FCS data of the fluorescent dyes ATTO 655 in water.

RESULTS AND DISCUSSION

Atto 655 and Atto-40-M2TM in Solution

As shown (Figure 1), the FCS curve obtained with the free Atto 655 dye in water can be adequately fit by Eq. (1) with a diffusion time (τ_D) of 73.6 μ s and a single dynamic component of 0.89 μ s (amplitude = 0.03). Similarly, the FCS curve measured with Atto-40-M2TM in ethanol can also be well described by Eq. (1) with a τ_D of 214 μ s and a single dynamic component of 0.54 μ s (amplitude = 0.02) (Figure 1). We attribute this dynamic component to triplet state formation as its relaxation time is within the time scale typical of the triplet lifetime of standard dyes.²² The small amplitude (0.02–0.03) of this component is also supportive of this assignment as it is known that Atto dyes have very low intersystem crossing yields.^{23,24} In the case of Atto-40-M2TM peptide, the lack of other dynamic components indicates that PET is not detectable in solution, suggesting that either an

encounter complex between the Trp residue and the dye is never formed, or the underlying PET dynamics are too slow/fast to be observed.²⁵

Liposome-Bound Atto-40-M2TM₄

To minimally perturb the native properties of the M2TM channel, the fluorescently labeled M2TM tetramers were assembled in the membrane of POPC/POPG (4/1) liposomes in a manner (see sample preparation) such that the large majority of tetramers contained either no or only one Atto-40-M2TM (such tetramer is hereafter referred to as Atto-40-M2TM₄). In addition, it was estimated that only approximately 10% of the liposomes contained one Atto-40-M2TM₄, and that the concentration of the Atto-40-M2TM₄ containing or fluorescent liposome was about 5–6 nM. As shown (Figure 2), the FCS curve obtained with Atto-40-M2TM₄ at pH 6.5 is quite different from that of Atto-40-M2TM monomer in solution (Figure 1). In fact, we found that at least four dynamic components were required in order to yield a satisfactory fit of these FCS data using Eq. (1) and a diffusion time of $2359 \pm 150 \mu\text{s}$. As shown in the Supporting Information (Figure S1), fitting these data to a simpler model yields an unsatisfactory fit. As indicated (Figure 2), these dynamic components are well separated in time and have the following respective time constants: $0.189 \pm 0.050 \mu\text{s}$, $2.66 \pm 0.50 \mu\text{s}$, $24.0 \pm 4.0 \mu\text{s}$, and $508 \pm 80 \mu\text{s}$. The $2.7 \mu\text{s}$ component most likely arises from triplet formation as the free dye exhibits a similar dynamic component and its amplitude shows a dependence on laser power (Figure S2 in Supporting Information). Moreover, results obtained from a control FCS experiment (Figure S3 in Supporting Information) indicated that those longer dynamic components are not due to the variability in the size of the liposome.

A simple estimate of the rotational diffusion coefficient of the liposome using the Einstein–Smoluchowski relation and the hydrodynamic radius of the liposome, which was determined based on its translational diffusion time (i.e., $\tau_D = 2400 \mu\text{s}$), indicates that the liposome should rotate on a time scale of $\sim 17 \mu\text{s}$. Thus, we attribute the $24.8 \pm 4.0 \mu\text{s}$ component to the rotational motion of the liposome. While either one or both of the remaining dynamic components could be attributed to PET, further experiments are required to determine their origin(s). Therefore, we performed control FCS experiments using a fluorescently labeled but Trp-free double mutant of M2TM, i.e., Leu40/Cys/Trp41/Phe, with the Atto 655 dye introduced at the Cys position (the resultant peptide is hereafter referred to as Atto-PheM2TM). Previous studies have shown that the Trp41/Phe mutant of M2TM exists in a tetrameric form in the POPC/POPG (4/1) membranes and conducts but lacks rectifying behavior.²⁶ In addition, the Trp to Phe mutation should eliminate PET as Trp41 is the only residue in M2TM that can quench the fluorescence of Atto 655.¹⁸ Similar to the Atto-40-M2TM₄ liposome samples, the Atto-Phe-M2TM containing tetramers (referred to hereafter as Atto-Phe-M2TM₄) were prepared so that each tetramer contains only one Atto-Phe-M2TM. As shown (Figure 3), the FCS curves obtained with Atto-PheM2TM₄ at different pHs can be well described by Eq. (1) with $\tau_D = 2192 \mu\text{s}$ and only three dynamic components, with time constants at $10 \pm 2 \mu\text{s}$ (due to liposome rotation), $1.0 \pm 0.2 \mu\text{s}$ (due to the triplet state), and $0.15 \pm 0.03 \mu\text{s}$. Thus, the absence of the $508 \mu\text{s}$ component, which was observed in the experiments with Atto-40-M2TM₄, indicates that this component is associated with PET. On the other hand, it is clear that the Trp to Phe mutation does not eliminate the nanosecond component, indicating that it has a different origin.

We attribute this nanosecond FCS component to internal fluctuations of the fluorescent reporter, either due to the rotation of the dye or the local dynamics of the backbone. Because the excitation light in the current case is linearly polarized, such motions would result in changes in the direction of the absorption transition dipole of the fluorophore and hence fluctuations in its fluorescence intensity. Of course, only those motions that are sufficiently slow are amenable to FCS detection.^{27,28} To verify this point, we measured the FCS curves

of a fluorescently labeled pH low insertion peptide (pHLIP)²⁹ in solution and membrane environments. The pHLIP peptide (the sequence used in the current study is: ACEQNPIYWARYADWLFTPLLLDLALLVDADEGTG) is designed to form a monomeric transmembrane α -helix at acidic pH.²⁹ In the current case, the fluorescence reporter, full name (TMR) is appended to the N-terminus of the peptide through the Cys residue. As shown (Figure 4), in solution the dynamics of the peptide and the dye are too fast to be measured by our FCS setup; however, when bound to POPC membranes as a transmembrane α -helix at pH 4,³⁰ a component of $0.064 \pm 0.030 \mu\text{s}$ is readily detectable. Taken together, these results support the notion that the local fluctuation dynamics of a dye reporter can be measured via FCS by using a linearly polarized excitation light.

Liposome-Bound Atto-40-M2TM₄ at Different pHs

Previous studies have suggested that the M2 channel undergoes a significant change in its structural equilibrium in response to a pH change from 7.0 to 5.0.^{10,11} Thus, we performed a series of FCS measurements using Atto-40-M2TM₄ in the pH range of 5.0–7.5. As shown (Figure 5), the resultant FCS curves, especially the nanosecond component, show a clear dependence on pH although the FCS signals are dominated by the diffusion component. To better determine the amplitudes of the local fluctuation and the PET components, we globally fit the FCS curves obtained at different pHs to the following model:

$$G(\tau, pH) = \frac{1}{N_{pH}} \left(\left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \left(\frac{1}{1 + \frac{\tau}{\omega^2 \tau_D}} \right)^{1/2} \right) \times \left(1 + A_{R,pH} \cdot \exp \left(-\frac{\tau}{\alpha \cdot (\tau_D)^3} \right) \right) \times \left(\frac{1 - \sum_{i=1}^m (T_{i,pH} - T_{i,pH} \cdot \exp(-\frac{\tau}{\tau_i}))}{1 - \sum_{i=1}^m T_{i,pH}} \right) \quad (2)$$

where the overall or global rotational time (τ_R) of the liposome, whose amplitude is $A_{R,pH}$, is assumed to be proportional to the third power of its translational diffusion time (τ_D).^{31,32} The three exponentials that were needed in the last term include those assigned to the triplet state, local fluctuation, and PET components. While N_{pH} , $T_{i,pH}$ and $A_{R,pH}$ were allowed to vary locally, all other parameters, including τ_D , τ_i , and α were globally restrained across all pHs during the fitting. As shown (Figure 5 and Figure S4 in the Supporting Information), this model fits the FCS curves well. Since the triplet state dynamics of Atto 655 as well as the translational and rotational diffusions of the liposome do not provide any information regarding the dynamics of the channel, in the following discussion we focus on the PET component ($\tau = 673 \pm 80 \mu\text{s}$) and the local fluctuation component ($\tau = 0.20 \pm 0.04 \mu\text{s}$).

PET Component

PET has been used to report peptide dynamics from the nanosecond to millisecond timescales.^{25,33} In our case, the PET reports a relaxation time of $673 \mu\text{s}$ whose amplitude exhibits a gradual decrease with decreasing pH (Figure 6). The latter is consistent with the notion that the M2 channel exists in at least two conformational states.^{14,15} The amplitude of the PET component represents the percentage of the peptide that is in the fluorescence dark state due to the PET interaction. In the current study, the fluorescence dark state induced by PET corresponds to the N-open state. At high pH (e.g., pH 7 and above), the N-open state is stabilized, in which the C-terminal regions of the four peptides are in closest proximity,^{10,11} yielding the largest PET. On the other hand, at low pH (e.g., pH 5) the C-open state is preferred in which the Trp41 moves away from position 40 due to the protonation of His37 residues that causes a rotamer flip of the Trp residues.¹¹ The rate of the PET component is comparable to the turnover rate (~ 1000 protons/sec) of the M2 channel measured at low pH.³⁴ Thus, it is possible that this component reports the actual opening/

closing of the M2TM channel. This idea is corroborated by recent simulations that suggest that the opening/closing motions of the channel occur at all pHs.^{14,15}

Local Fluctuation Component

The amplitude of the internal fluctuation component shows a drastic decrease between pH 6.5 and 6.0 (Figure 6), a pH range that has been shown to activate the M2 channel.³⁴ Thus, this coincidence suggests that this FCS component is a useful probe of the shift of the conformational equilibrium of the M2TM channel. In particular, this component exhibits a relatively large amplitude when the M2TM tetramer favors the N-open state at high pH, whereas it practically disappears when the C-open state is favored at low pH. It appears from a fitting of the intensities to a two state model that this transition is consistent with a published pKa value (6.3) of the histidine residue.³⁵

There are two possible reasons that could lead to the disappearance of this component at low pH. The first possibility is that the dye fluctuates too quickly (faster than 40 ns) to be resolved by our FCS setup when the channel is in its C-open state. In support of this, previous studies have shown that at low pH the backbone of M2TM becomes more dynamic,^{10,36} and that Leu40 is less restricted by other chains in the tetramer and is also more accessible to the interior of the channel.¹¹ The other possibility is that the motion of the dye becomes too restricted to induce any measurable fluctuations in its fluorescence. To better understand the origin of the pH-dependence of this local component, we performed further control FCS experiments using a different fluorescently labeled M2TM peptide, with the Atto 655 dye introduced at position 32 using the Ile32/Cys mutant (the M2TM tetramer containing this peptide is hereafter referred to as Atto-32-M2TM₄). Since Ile32 is close to the middle of the channel with its sidechain facing the lipids, it is expected that the local fluctuation component of Atto-32-M2TM₄ will be less sensitive to pH. In addition, it is expected that the PET component will disappear as in this case the Trp residues are too far from the dye to show any appreciable fluorescence quenching. As shown (Figure 7), the FCS curves obtained with Atto-32-M2TM₄ indeed lack the PET component, while the local dynamic component is present in the entire pH range studied. Taken together, these data further illustrate that this dynamic component is sensitive to local environment and that, when the dye reporter is placed at an appropriate position, it can be used as a valuable probe of the M2TM tetramer conformational equilibrium and dynamics.³⁷ This statement is further corroborated by the Atto-PheM2TM₄ results, which show that this FCS component is present in the entire pH range studied, suggesting that deletion of the key His-Trp interactions²⁶ makes the tetramer adopt a conformation that is similar to the closed state or a cylindrical shaped structure of the M2TM channel.¹¹

Effect of Amantadine

The above results suggest that the 0.2 μ s component observed with Atto-40-M2TM₄ is a useful probe of the pH-induced conformational shift of the M2TM tetramer, between the N-open and C-open states. Thus, in the following experiments we use it to probe how drug binding affects the conformational equilibrium of the M2TM channel. As shown (Figure 8), binding of amantadine to Atto-40-M2TM₄ does not significantly change the FCS curves. Similar to that observed for drug free Atto-40-M2TM₄, the amplitude of the PET component ($\tau = 557 \pm 80 \mu$ s) shows a minor decrease with decreasing pH from 7.5 to 5.0 and the amplitude of the local fluctuation component ($0.16 \pm 0.04 \mu$ s) exhibits a large decrease between pH 6.0–5.0 (Figure 9). The increase in the rate of the PET process upon drug binding is consistent with the notion that the drug will affect the His-Trp interactions and, thus, the Trp dynamics. However, taken together, these results suggest that drug binding does not prevent the M2TM tetramer from undergoing the underlying shift in conformational equilibrium upon protonation of the His residues, although, according to a

fitting, the transition seems to occur at a lower pH, which is expected given that the pK_a of the His residues shifts to 5.4 in the presence of amantadine.³⁸ In other words, these results are consistent with the crystal structure of Stouffer *et al.* which shows that amantadine binds to the center of the M2TM channel and, as a result, physically occludes the pore but does not inhibit the pH-appropriate conformational equilibrium shift of the channel.^{11,12} Results obtained with another drug rimantadine (data not shown) also corroborate this conclusion.

CONCLUSIONS

In summary, we employ fluorescence correlation spectroscopy (FCS) to investigate the conformation and conformational dynamics of a truncated version of the Influenza A M2 proton channel, M2TM, in the pH range of 5.0–7.0 and with and without the presence of inhibition drugs. Our results show that both the photoinduced electron transfer (PET) process from Trp to the dye reporter and the local fluctuation motions of the dye can be used to probe changes in structure and/or dynamics of the membrane-bound M2TM tetramer. In particular, our data support a proposed inhibition mechanism that involves the drugs binding to the interior of the channel and physically occluding the pore.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representatives FCS curves obtained with the free dye, Atto 665, in buffer solution (blue) and Atto-40-M2TM in ethanol (green). The red lines represent the best fit of these data to Eq. (1) with $m = 1$ and the following parameters: $\tau_D = 73.6 \mu\text{s}$, $\tau_I = 0.89 \mu\text{s}$, $T_I = 0.03$ for the free dye and $\tau_D = 214 \mu\text{s}$, $\tau_I = 0.54 \mu\text{s}$, $T_I = 0.02$ for the peptide.

Figure 2.

A representative FCS curve (blue) obtained with Atto-40-M2TM₄ at pH 6.5. The solid red line represents the best fit of these data to Eq. (1) with $m = 4$. The dashed red line represents the diffusion component, the green line represents the non-diffusional portion of the FCS curve (i.e., $G(\tau) - \text{diffusion component}$), and the black line corresponds to the difference between the red solid line and the red dashed line. Also shown in the top panel are residuals of the fit.

Figure 3.

Representative FCS curves obtained with Atto-Phe-M2TM₄ at different pHs, as indicated. The red line represents the best fits of the FCS data to Eq. (1) with $m = 3$. The green line represents the non-diffusional portion of each FCS curve (i.e., $G(\tau) - \text{diffusion component}$), and the black line corresponds to its fit (i.e., Fit of $G(\tau) - \text{diffusion component}$).

Figure 4. Representative FCS curves obtained with pHLIP in the presence (blue) and absence (green) of membranes at pH 4.

Figure 5. Representative FCS curves (blue) obtained with Atto-40-M2TM₄ at different pHs, as indicated. These data have been offset for clarity. Red lines represent the global fits of these data to Eq. (2) with $m = 3$. The fitting parameters and residuals of the fit are given in the Supporting Information (Table S1 and Figure S5).

Figure 6. Amplitudes of the PET and local dynamic components of Atto-40-M2TM₄ versus pH. Errors were estimated from multiple measurements.

Figure 7.

Representative FCS curves obtained with Atto-32-M2TM₄ at different pHs, as indicated. Red lines represent the global fits of these data to Eq. (2) with $m = 2$, $\tau_1 = 0.092 \mu\text{s}$ (with $T_1 = 0.16, 0.20$, and 0.21 for pH = 7.0, 5.5 and 5.0, respectively) and $\tau_2 = 0.57 \mu\text{s}$ (with $T_2 = 0.10, 0.13$, and 0.09 for pH = 7.0, 5.5 and 5.0, respectively). The green line represents the non-diffusional portion of each FCS curve (i.e., $G(\tau) - \text{diffusion component}$), and the black line corresponds to its fit (i.e., Fit of $G(\tau) - \text{diffusion component}$). The residuals of the fit are given in the Supporting Information (Figure S4).

Figure 8.

Representative FCS curves obtained with drug-bound Atto-40-M2TM₄ at different pHs, as indicated. These data have been offset for clarity. Red lines represent the global fits of these data to Eq. (2) with $m = 3$. The fitting parameters and residuals are given in the Supporting Information (Table S2 and Figure S6).

Figure 9.
Amplitudes of the PET and local dynamic components obtained with drug-bound Atto-40-M2TM₄ versus pH, as indicated.