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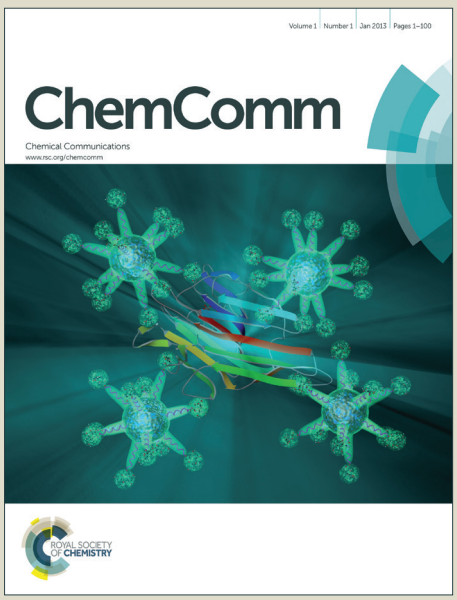

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β-(1-azulenyl)-L-alanine – a functional probe for determination of pK_a of histidine residudes

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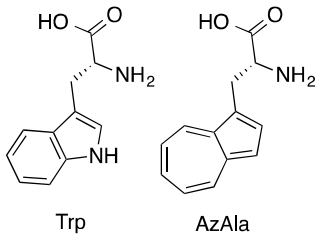
β-(1-azulenyl)-L-alanine (AzAla) can be incorporated into the influenza A virus M2 proton channel. AzAla's sensitivity to protonation state of the nearby histidines and the lack of environmental fluorescence dependence allows for direct and straightforward determination of histidine pK_a values in ion channels.

Fluorescent probes have been enormously useful in a large variety of biophysical and biochemical studies.[1] They provide a straightforward and inexpensive way for monitoring protein folding, protein-ligand and protein-protein interactions.[2] Intrinsic fluorescent probes are of particular value for such experiments, as they do not disturb the native properties of the object of the study. Tryptophan (Trp) is particularly useful due to its ability to be excited independently of most other intrinsic chromophores and its low abundance in proteins that simplifies data analysis.[3] Moreover, tryptophan's sensitivity to the environment and locally present quenchers offers a lot of information about probe localization. At the same time some of Trp's advantages could be its Achilles heels: Trp's sensitivity to *both* the local environment and quenchers inherently present in proteins (histidine, methionine, etc.) can often convolute the analysis of the fluorescence data.



Scheme 1. Structures of tryptophan (Trp) and β-(1-azulenyl)-L-alanine (AzAla).

Recently, we have reported that fluorescence profile of β-(1-azulenyl)-L-alanine (AzAla, Scheme 1), a pseudoisosteric analog of Trp, does not depend on the local environment but it does responds to weak intrinsic quenchers.[4] This unique feature allows for studying the subtle detail of protein-protein and protein-peptide interactions in a minimally invasive way. We have previously shown that AzAla does not perturb protein-peptide interactions and in the present study we put this Trp analog to even more stringent test – determine whether replacing Trp with AzAla in ion channels preserves its functionality.

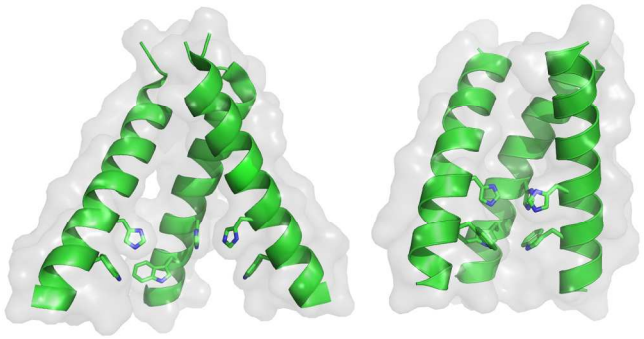
Ion channels represent one of the most common classes of membrane proteins. Despite their immense fundamental and practical importance, ion channels are notoriously hard to study – inherently, they have both soluble and membrane domains, that greatly limits applicability of many standard biophysical techniques. Subtle structural changes observed during ion conductance are often masked by the effects of the highly inhomogeneous lipid bilayer. Structural characterization by X-ray crystallography and solid state NMR (ssNMR) has been incredibly useful in understanding the mechanism of ion channel function, but these techniques place stringent requirements on the sample – crystals are needed for crystallography, expensive isotopic labelling is necessary for NMR. While offering lower resolution in terms of structural and functional information, fluorescent probes allow for straightforward measurements regardless of protein's size. Moreover, intrinsic and translationally incorporated fluorophores can be potentially used for *in vivo* studies.

An ideal probe for fluorescent studies of complex membrane-associated proteins has to satisfy two key conditions: first, the probe must preserve the original function to the maximal possible extent; and, second, the probe has to provide a distinct readout that does not require complex data processing. Due to its favourable spectroscopic properties, AzAla can potentially meet both conditions.

AzAla has been shown to preserve binding properties of various peptides,[5] but no studies have been conducted where AzAla replaced key tryptophan residues intimately involved in protein's function. In order to establish the extent to which AzAla perturbs the native function of ions channels we introduced it into M2 proton channel of influenza A virus. Influenza A M2 proton channel (M2) is an integral membrane protein that is essential for viral entry into the

cell. M2 is a major pharmaceutical target and therefore it has been extensively biophysically characterized.[6] This 96-residue protein oligomerizes into a tetramer in lipid bilayers to create a functional channel. It has been shown that the transmembrane (residues 22-46) domain of this protein (M2TM) is capable of maintaining the function in the absence of soluble domains. M2TM has been structurally characterized by X-ray crystallography,[7] solution[8] and solid state NMR.[9] Thus M2TM presents an excellent model system to test the effects of AzAla on protein function and its applicability to measure pK_a of histidine in the membrane environment.

The X-ray structure of M2TM is shown in Figure 1. The unique tryptophan of the sequence (Trp41) is located in the pore-lining region and is critical for ion channel's function as it is responsible for gating – mutating Trp41 permits the reverse outward current.[10] His37 that is primarily responsible for proton conductance lies one helical turn above Trp41. Two mechanisms have been proposed for proton conductance: Pinto *et al.* postulated a ring-flip cycle of protonation and deprotonation in "His-box" formed by a tetrad of the four histidine residues; Cross *et al.* proposed that the His37 tetrad partitions into a dimer of dimers stabilized by low-barrier hydrogen bonds.[11] Recent experimental and theoretical studies suggest that both mechanisms may play a role in proton conductance.[12]



Sequence: SSDPLVVAASIIGIL**H**^{37}LIL**W**^{41}ILDRL

Figure 1. X-ray crystal structure of M2TM showing the histidine tetrad and the gating Trp41 at pH 7.3 (closed state, left)[7b] and pH 6.5 (open state, right).[7a] One helix of the tetramer and the side chains other than His37 and Trp41 are omitted for clarity.

Characterization of physico-chemical properties of His37 is of paramount importance for mechanistic studies of proton conductance. Much effort has been dedicated to establish the pK_a values for the His37 tetrad. pK_a of the His37 in monomeric M2 protein has shown to be 6.8 in dodecylphosphatidylcholine micelles, consistent with the pK_a value of the side chain of the isolated histidine residue.[13] Early studies of M2 channel in D_2O using UV resonance Raman spectroscopy established a single pK_a value of 5.7 for all four histidine residues.[14] Subsequent studies using ssNMR yielded values of 8.2 ± 0.2, 8.2 ± 0.2, 6.3 ± 0.3, and < 5.0 for the four sequential protonation steps.[11] More recently, reconstitution of the protein in lipid films closely resembling those of the virus allowed for resolution of the first two pK_a values: 7.6 ± 0.1, 6.8 ± 0.2, 4.9 ± 0.3, and 4.2 ± 0.6.[15] It should be noted that in all cases the pK_a's were obtained from the limited number (5-7) of pH conditions due to complexity of the state-of-the-art techniques employed in these studies. Different results obtained by various groups further underscore the difficulty of reliably obtaining pK_a values in complex protein assemblies.

We set to explore whether Trp41AzAla mutation in M2TM can be used to determine pK_a values of the histidine residues of the tetrad with a very basic pH titration experiment. In order to test the ability of AzAla to determine pK_a values of the nearby histidine residues we looked at fluorescence of N-acetyl-(azulenylalanine)-propylamide (NAAzAP) as a function of pH in the presence of imidazole and histidine (ESI†, S5). The observed pH profile of fluorescence in universal buffer can be easily fit to a single step deprotonation model to obtain the pK_a's of 6.0 ± 0.1 for histidine and 6.9 ± 0.1 for imidazole, reproducing the literature values perfectly.

Having established the overall feasibility of quantitatively characterizing protonation equilibria, we tested whether mutation of highly conserved Trp41 to AzAla impacts conductance of protons by M2TM. We have compared proton flux of the full length M2 protein, M2TM and M2TM Trp41AzAla embedded in the lipid vesicles generally following the previously established methodology.[16] M2TM and M2TM Trp41AzAla were prepared by solid phase peptide synthesis optimized for hydrophobic sequences[17] and the full length M2 protein was expressed and purified using previously published procedure.[18] M2 constructs were then reconstituted in lipid vesicles mimicking the composition of viral membrane (4:2:1 POPC:POPG:cholesterol) with the buffer containing only potassium ions. The buffer on the exterior was then exchanged to replace K^+ with Na^+. Subsequent addition of valinomycin, a potassium ionophore, results in leakage of K^+ from the vesicles. The resulting charge gradient initiates acidification of the vesicle interior, which is monitored using pyranine, a fluorescent pH sensor (Figure 2).

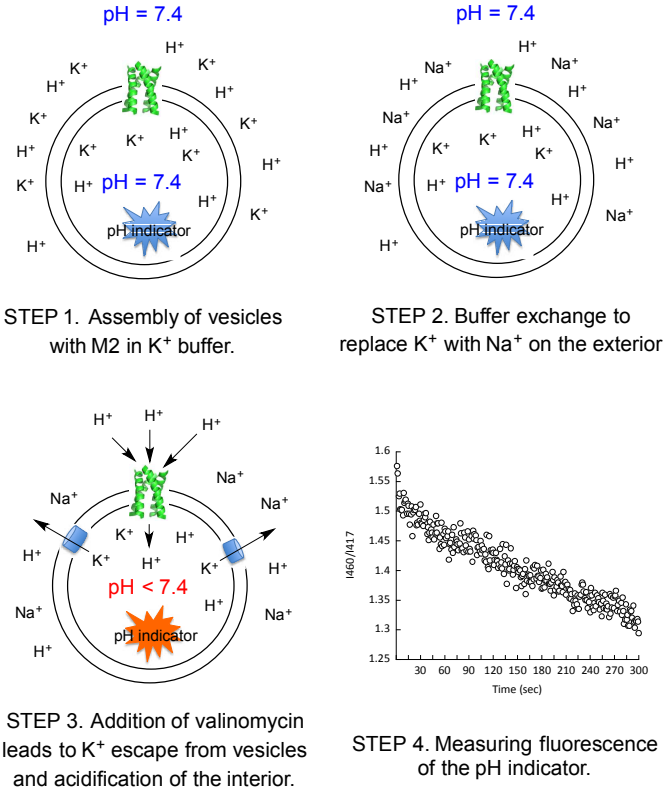


Figure 2. Overview of the proton conductance studies. Detailed experimental procedure is given in ESI.

In line with previous studies[19] we found the proton flux induced by M2 to be on the order of 6 protons*s^{-1}*tetramer (Table 1). Increased apparent flux for the transmembrane peptide compared to the wild type protein also observed in the previous studies was

attributed to either orientational preferences or genuine differences in conductivity.[20] Next, we mixed M2TM with M2TM Trp41AzAla in 3:1 ratio in an attempt to create a functional tetramer with a single fluorescent label.

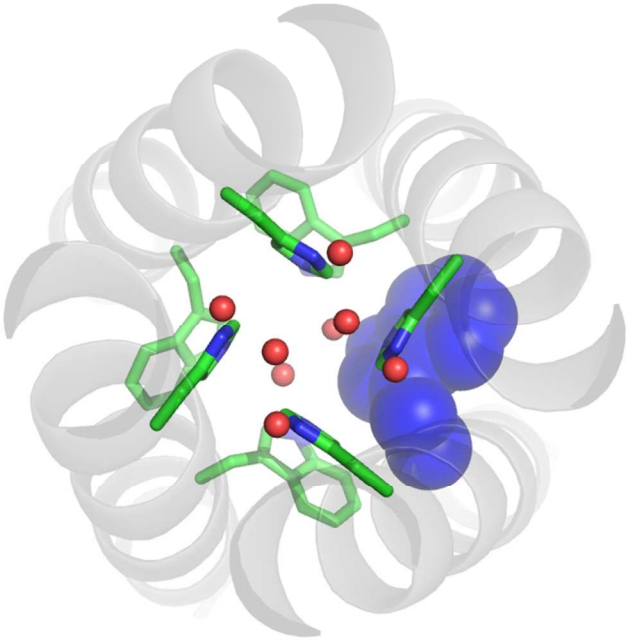


Figure 3. A model of a tetramer comprised of M2TM and M2TM Trp41AzAla in a 3:1 ratio based on a high resolution crystal structure of M2TM (PDB code 3LBW from ref. 7a). The AzAla residue is shown in blue spheres, side chains of all residues except for His37 and Trp41 are omitted for clarity, water molecules that form a hydrogen-bonding network that effectively links all four histidines are shown in red spheres.

Introduction of M2TM Trp41AzAla resulted in minimal change of the proton flux (Table 1) suggesting that the mutant associates with the wild type peptide without significant loss in function and single AzAla residue exhibits no significant detrimental effect on the properties of the M2 ion channel.

Table 1. Proton flux shown by various M2 variants.

Protein	Proton flux (protons*s^{-1}*$tetramer^{-1}$)
M2 full length	6 ± 1
M2TM	18 ± 7
M2TM:M2TM Trp41AzAla (3:1)	15 ± 1

In the wild type M2TM channel, Trp41 lies one helical turn away from His37. Trp41 forms a "proton gate", which blocks proton diffusion from the inside of the virus. When assembled in a functional tetramer, all four histidines are connected through multiple hydrogen bonds formed by water molecules located in the channel. The resulting "water box" is linked to the tryptophans via cation-π interactions (Figure 3). Thus sequential protonation results in concerted changes in the histidine tetrad, which can be sensed by AzAla. Therefore we proceeded to study the fluorescence profile of AzAla in the M2 channel as a function of pH. AzAla can be excited independently of all other fluorophores in the channel at 342 nm. Ability to be excited at this relatively high wavelength distinguishes AzAla from all other pseudoisosteric analogs of tryptophan. The pH profile of fluorescence M2TM:M2TM Trp41AzAla (3:1) tetramer reconstituted in a phospholipid bilayer is shown in Figure 4.

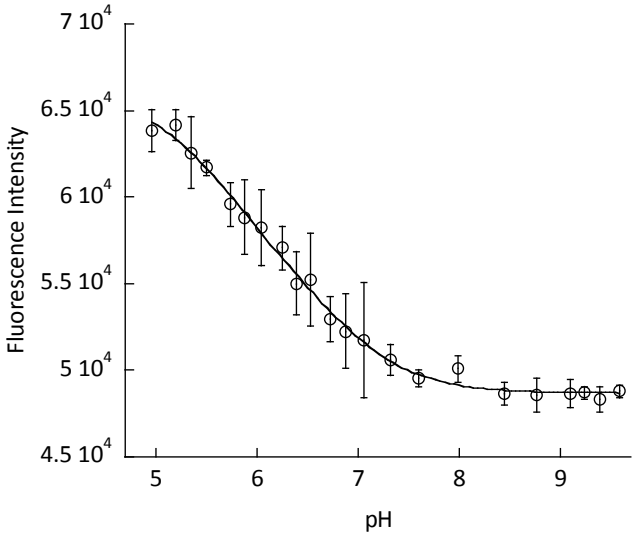


Figure 4. pH dependence of the fluorescence of M2TM:M2TM Trp41AzAla (3:1) tetramer; λ_{ex} = 342 nm, λ_{em} = 382 nm.

The pH profile can be best fitted to two protonation steps with pK_a values of 6.9 ± 0.2 and 5.6 ± 0.3. These values are in good agreement with the values found by Hong *et al.* using solid state NMR for the second and third protonation steps, processes known to induce the largest reorganization of the channel.[15] While fluorescence pH profile shown by AzAla does not allow for unambiguous determination of *all* pK_a values in M2TM, it can pinpoint the protonation step occurring at the histidine closest to the probe and/or the protonation step that leads to the largest structural reorganization in the channel. It should be noted that multiple pK_a values obtained from the solid state NMR studies are results of deconvolution of chemical shift changes of multiple nuclei together with functional information. Simple and inexpensive fluorescence results obtained using AzAla will undoubtedly aid these complex studies. With the very recent advent of a methodology to translationally introduce AzAla into proteins using *L. lactis*, an excellent expression host for recombinant production of membrane proteins,[21] we expect this probe to gain widespread use as a chemical biology tool.

Conclusions

30 years ago Hudson *et al.* postulated[22] that substitution of the indole moiety in tryptophan with azulene will produce a fluorescent probe with unique properties. However, until recently synthetic difficulties and absence of the path for biosynthethic incorporation left AzAla overshadowed by other tryptophan mimics. We have previously established that, unlike tryptophan or all other known tryptophan mimics, AzAla's fluorescence doesn't depend on the local environment. At the same time AzAla's fluorescence can be quenched by mild intrinsic quenchers (Met, His). This feature is invaluable for

studies of membrane proteins, where small conformational changes can greatly affect the local environment of the fluorophore. In this work we showed that AzAla's utility extends even further: it can replace tryptophan in the influenza A M2 proton channel. Moreover, it can serve as a reporter of the protonation state of the neighbouring histidine residue. With new methodology to introduce AzAla into proteins *in vivo* we expect this fluorescent probe to gain widespread use as a chemical biology tool.

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Notes and references

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† Electronic Supplementary Information (ESI) available: detailed experimental protocols for measuring proton flux and fluorescence studies, peptide synthesis and protein expression. See DOI: 10.1039/c000000x/

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