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Probing the polarity and water environment at the protein-peptide binding interface using tryptophan analogues

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ABSTRACT

7-Azatryptophan and 2,7-diazatryptophan are sensitive to polarity changes and water content, respectively, and should be ideal for studying protein-protein and protein-peptide interactions. In this study, we replaced the tryptophan in peptide **Baa** (LKWKKLLKLLKKLLKLG-NH_2) with 7-azatryptophan or 2,7-diazatryptophan, forming (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa**, to study the calmodulin (**CaM**)-peptide interaction. Dramatic differences in the (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa** fluorescence properties between free peptide in water and calmodulin-bound peptide were observed, showing a less polar and water scant environment at the binding interface of the peptide upon calmodulin binding. The affinity of the peptides for binding **CaM** followed the trend **Baa** (210 ± 10 pM) < (7-aza)Trp-**Baa** (109 ± 5 pM) < (2,7-aza)Trp-**Baa** (45 ± 2 pM), showing moderate increase in binding affinity upon increasing the number of nitrogen atoms in the Trp analogue. The increased binding affinity may be due to the formation of more hydrogen bonds upon binding **CaM** for the Trp analogue with more nitrogen atoms. Importantly, the results demonstrate that (7-aza)Trp and (2,7-aza)Trp are excellent probes for exploring the environment at the interface of protein-peptide interactions.

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1. Introduction

Tryptophan (Trp) is the most common natural amino acid fluorophore in biophysics to monitor changes in protein conformations upon substrate binding and/or protein-protein interaction. This is because of the long absorption wavelength and high emission yield for the indole moiety. However, proteins usually contain more than one Trp residue, making it difficult (if not impossible) to assign the changes in fluorescence signal to a particular site definitively. This makes interpreting the spectral changes that result from specific protein interactions complicated and difficult. Nonetheless, the Trp analogue 7-azatryptophan ((7-aza)Trp), Scheme 1(a)) has been used as an alternative of Trp to probe protein structure and dynamics [1–3]. (7-aza)Trp exhibits an even longer absorption spectral onset compared to Trp and polarity-sensitive emission properties that can probe the surrounding environment [4,5]. Recently, we reported a new Trp analogue, (2,7-aza)Trp [1]; the emission property of which is not sensitive to the environment polarity. Instead, (2,7-aza)Trp exists predominantly as the N(1)-H isomer with a minor N(2)-H isomer in neutral aqueous solution. The N(1)-H undergoes a water-catalyzed N(1)-H→N(7)-H proton transfer in the excited state, resulting in the N(7)-H tautomer with green emission (Scheme 1(b)). Both the N(1)-H (350 nm) and the N(7)-H tautomer (500 nm) emissions are observed in water rich environments. In contrast, only the N(1)-H emission at 350 nm is observed under water scant conditions. The ratiometric emission and the associated relaxation dynamics may also provide certain clues for specific water-Trp interactions in proteins. The N(2)-H isomer only exists in bulk water and generally disappears in protein environments with microsolvated water molecules, unless in the presence of specific N(2)-H…acceptor H-bonds [2]. (2,7-aza)Trp would thus be ideal for probing the water microsolvation surrounding Trp in proteins.

We herein demonstrate the strategy of combining (7-aza)Trp and (2,7-aza)Trp in probing protein-peptide interactions (Scheme 1(c)). We chose to study calmodulin (**CaM**), which is a relatively small but crucial protein (148 residues; approximately 17 kDa; more than 0.1% of total protein in cells). **CaM** plays a key role in intracellular signal transduction by folding in the presence of calcium as well as binding and activating enzymes [6]. **CaM** binds four calcium ions with micromolar affinity in response to a

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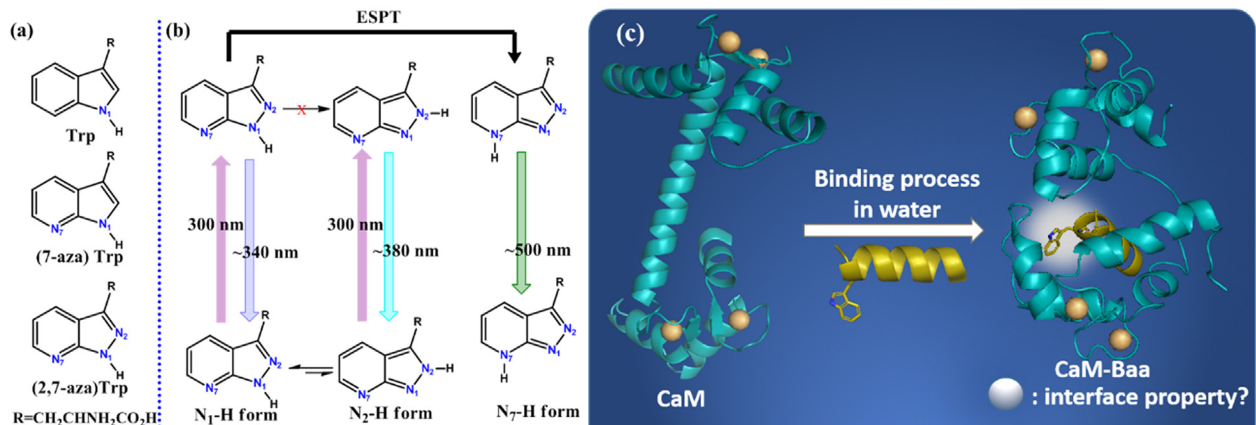

Scheme 1. (a) The chemical structures of Trp, (7-aza)Trp and (2,7-aza)Trp. (b) The ground-state equilibrium between N(1)-H and N(2)-H for (2,7-aza)Trp in neutral water and the water-catalyzed N(1)-H → N(7)-H proton transfer in the excited state for (2,7-aza)Trp. (c) Qualitative diagram of **CaM** and **Baa** binding process in water. Note the **CaM** structure was isolated from free **CaM** (PDB ID: 3CLN) and **CaM** bound **Baa** (PDB ID: 2BE6, in which CaV1.2 IQ domain of 2BE6 is replaced by **Baa**.

variety of extracellular signals that alter cellular calcium levels. Calcium-binding leads to conformational changes that enable **CaM**-Ca^{2+} complex to recognize and bind target proteins with high affinity ($k_d = 10^{-7}$ to 10^{-11} M) [7]. The crystal structure of **CaM** with four bound Ca^{2+} -ions (**CaM**-Ca^{2+} complex) revealed two globular domains (N- and C-terminal domains; also called the N-lobe and the C-lobe), with each containing two EF-hand type Ca^{2+}-binding sites [8–11]. The two domains are connected by a linker helix of approximately eight turns that shows some conformational flexibility in solution, as revealed by NMR and small-angle X-ray scattering (SAXS) experiments [12–15]. Three-dimensional structures of calmodulin in complex with high-affinity peptidic substrates have been determined [16–18]. These peptides correspond to the calmodulin-binding regions of different protein kinases. The high-affinity binding of calmodulin to both peptidic and non-peptidic substrates can be abolished by addition of calcium chelators, making this system an interesting candidate for biotechnological applications [19]. The **CaM**–peptide interaction is a convenient model for determining how proteins associate and dissociate, which is fundamental to molecular recognition and signal transduction activation processes. O'Neil & DeGrado [20] have studied this system by strategically designed peptides, which form a putative amphiphilic helix and bind to calmodulin with sub-nanomolar dissociation constants (k_d). With the three-dimensional structures available [16–18], it is now possible to extend this research further by using the **CaM**–peptide complex system to investigate the polarity and water environment at the interface of the high-affinity interaction. In this article discussed below, for convenience, we denote the calcium-contain calmodulin as **CaM**.

Our peptides were based on peptide **Baa** (LKWKKLLKLLKKLLKLG-NH_2) [21], which embodies the quintessential elements of the structural feature but has minimal sequence homology to any of the peptides to be mimicked. Moreover, **Baa** contains only one Trp residue to avoid complication in data interpretation (when multiple Trp residues are present). **Baa** binds **CaM** with a dissociation constant (k_d) of 210 ± 10 pM and **Baa** binds preferentially with Trp near the C-terminal half of the protein [22]. The Trp in **Baa** was replaced with (7-aza)Trp and (2,7-aza)Trp, forming (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa**. Since the emission of (7-aza)Trp and (2,7-aza)Trp is sensitive to their surrounded polarity and water microsolvation, respectively (vide supra), the corresponding spectral variations should provide valuable interface information of the interface amid binding. Moreover, since Trp plays a role in the **CaM-Baa** interaction, how these (7-aza)Trp and (2,7-aza)Trp analogues affect the binding affinity of the peptides for **CaM** is of fundamental interest.

2. Materials and methods

2.1. General section

The bovine brain calmodulin used in the study was purchased from Sigma-Aldrich and used without further purification. Millipore water (18 MΩ cm) was used to prepare all aqueous solutions. All other amino acids used in the peptide synthesis were purchased from Novabiochem. The basic, amphiphilic α-helix (**Baa**) were synthesized by a standard Fmoc-protocol and purified by preparative RP-HPLC. The identity of the product was verified by MALDI-TOF mass spectrometry.

2.2. Synthesis of Fmoc-(2,7-aza)Trp and Fmoc-(7-aza)Trp

After dissolving (2,7-aza)Trp or (7-aza)Trp in water, 2 eq sodium bicarbonate was added with stirring at room temperature. The resulting solution was then allowed to cool down to 5 °C and then Fmoc-OSu (1.5 eq) was added slowly as a solution in dioxane. The resulting mixture was stirred at 0 °C for 1 h and allowed to warm back to room temperature overnight. Water was then added, and the aqueous layer was extracted twice with EtOAc. The organic layer was then back extracted twice with saturated sodium bicarbonate solution. The combined aqueous layers are acidified to a pH ~ 1 with 10% HCl, followed by the extraction with EtOAc three times. The combined organic layers were dried (sodium sulfate) and concentrated in vacuo. The resulting residue was purified by flash chromatography (SiO_2) [23].

The ^{1}H NMR (400 MHz) of Fmoc-(7-aza)Trp in d-DMSO, δ (ppm): 4.14-4.21 (4H, m), 6.99-7.01 (1H, m), 7.22–7.30 (3H, m), 7.36-7.40 (2H, m), 7.53-7.64 (2H, m), 7.70 (1H, d, J=7.6 Hz), 7.85 (2H, d, J=8 Hz), 7.97 (1H, d, J=8 Hz), 8.16 (1H, d, J=4.4 Hz), 11.38 (1H, s). The ^{1}H NMR of Fmoc-(2,7-aza)Trp in d-DMSO, δ (ppm): 4.10-4.19 (3H, m), 4.39-4.45 (1H, m), 7.07-7.10 (1H, m), 7.21-7.31 (2H, m), 7.38 (2H, t, J=7.6 Hz), 7.59 (2H, t, J=8 Hz), 7.79 (1H, d, J=8.4 Hz), 7.85 (2 H, d, J=7.6 Hz), 8.26 (1H, d, J=6.4 Hz), 8.45 (1H, d, J=2.8 Hz), 12.79 (1H, br), 13.35 (1H, s). (see Fig. S1).

2.3. Synthesis of Baa

(Ac-Leu-Lys-Trp-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly-NH_2).

The peptide was synthesized using 504 mg (0.10 mmol) of Fmoc-PAL-PEG-PS resin. The synthesis gave 646 mg of resin (52.8% in yield). The large scale cleavage yielded 150 mg of crude peptide. The peptide was purified by preparative RP-HPLC using a C_4 (PLG34–45) and a C_{18} column (PLG37–51) to 96.9% purity. The amount of pure peptide was 14.0 mg. Retention time on analytical RP-HPLC was 47.8 min. The identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{105}H_{190}N_{26}O_{18}$ $[MH]^+$:2105.483; observed: 2105.990. The α-helix structure of **Baa** was identified by the CD spectrum (see Fig. S2 of SI).

2.4. Synthesis of (7-aza)Trp-Baa

(Ac-Leu-Lys-(7-aza)Trp-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly-NH_2).

The peptide was synthesized using 207 mg (0.05 mmol) of Fmoc-PAL-PEG-PS resin. The synthesis gave 329 mg of resin (99% in yield). The large scale cleavage yielded 83 mg of crude peptide. The peptide was purified by preparative RP-HPLC using a C_4 (PLG31–41) and a C_{18} column (PLG36–46) to 90.4% purity. The amount of pure peptide was 15 mg. Retention time on analytical RP-HPLC was 47.6 min. The identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{104}H_{189}N_{27}O_{18}$ $[MH]^+$:2105.47; observed: 2105.635. The α-helix structure of (7-aza)Trp-**Baa** was identified by the CD spectrum (see Fig. S2).

2.5. Synthesis of (2,7-aza)Trp-Baa

(Ac-Leu-Lys-(2,7-aza)Trp-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly-NH_2).

The peptide was synthesized using 206 mg (0.050 mmol) of Fmoc-PAL-PEG-PS resin. The synthesis gave 327 mg of resin (99% in yield). The large scale cleavage yielded 80 mg of crude peptide. The peptide was purified by preparative RP-HPLC using a C_4 column (PLG31–41) and a C_{18} column (PLG36–46) to 90.0% purity. The amount of pure peptide was 13 mg. Retention time on analytical RP-HPLC was 47.8 min. The identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{103}H_{188}N_{28}O_{18}$ $[MH]^+$:2106.473; observed: 2104.815. The α-helix structure of (2,7-aza)Trp-**Baa** was identified by the CD spectrum (see Fig. S2).

2.6. Steady-state and time-resolved fluorescence spectroscopy

Steady-state absorption and emission spectra were recorded on a Hitachi (U-3310) spectrophotometer and an Edinburgh (FS920) fluorimeter, respectively. Both wavelength dependent excitation and emission response of the fluorimeter had been carefully calibrated. The concentration of these peptides or **CaM** was determined spectrophotometrically by using the absorption extinction coefficient for the sole Trp residue in the peptide (see Fig. S3) and tyrosine (Tyr) residues in the **CaM**. The binding of calmodulin to **Baa**, (7-aza)Trp-**Baa** or (2,7-aza)Trp-**Baa** was monitored by Trp or Trp analogues fluorescence at 25 °C. A stock solution of **CaM** (0–6 μM final concentration) was titrated into a 1 μM solution of **Baa**, (7-aza)Trp-**Baa** or (2,7-aza)Trp-**Baa**. Corrections to the intensity measurements were made for dilution during the titration. The buffer was 5 mM Tris–HCl, pH 7.5, 1.0 mM $CaCl_2$. To ensure equilibration of the mixture, the sample was constantly stirred for 1 min and incubated for 10 min at 25 °C before measurement. The fluorescence spectrum for each sample is an average of three replica. In this approach, our peptide sequence is the same as that reported by DeGrado et al., namely the peptide, **Baa**, which interacts with **CaM** with 1:1 stoichiometry. Accordingly, the one-site biding equation is suitable to deduce the dissociation constant. The experimental binding curves were then fitted by the following equation using a nonlinear regression routine:

$$\Delta I = \frac{\Delta I_{max}\cdot[CaM]}{[CaM] + k_d}$$

where ΔI is the change in fluorescence intensity of (2,7-aza)Trp-**Baa** or (7-aza)Trp-**Baa** induced by **CaM**. On the one hand, the fluorescence intensity of (7-aza)Trp-**Baa/CaM** was monitored at 358 nm, which is much sensitive to the changes of polarity. On the other hand, the fluorescence intensity for (2,7-aza)Trp-**Baa/CaM** was monitored at 380 nm that is sensitive to the water surrounding. ΔI_{max} is the maximal change in (2,7-aza)Trp-**Baa** or (7-aza)Trp-**Baa** fluorescence saturated with **CaM**. To obtain the limiting value for the binding affinity, we used the equivalent [CaM]/[Baa] unit to represent the concentration change and compared the k_d value with that deduced by DeGrado et al. using competition method.

Nanosecond (ns) lifetime studies were performed with a time-correlated single-photon counting (TCSPC, Edinburgh FL900), in which a hydrogen-filled lamp was used as the excitation source. The emission decays were analysed by the sum of exponential functions with system response function incorporated, which allow partial elimination of instrument time broadening and renders a temporal resolution of ~300 ps.

To achieve better time resolution, studies were also performed using a TCSPC system (Edinburgh OB-900L) coupled with a microchannel plate (MCP) detector and an excitation light from the third harmonic generation (THG, at 310 nm) of pulse-selected femtosecond laser pulses at 930 nm (82 MHz, 90 fs, Tsunami, Spectra-Physics). The temporal resolution, after partial removal of the instrumental time broadening, was ~15 ps. For both time-resolved measurements the fluorescence was collected at a right angle with respect to the pump beam path and passed through a polarizer, which was located in front of the detector (54.7°) to eliminate anisotropy.

3. Results and discussion

The fluorescence spectral changes are used to investigate the **CaM** binding with (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa**. Similar to that of 7-azaindole [24,25], the fluorescence of (7-aza)Trp has been reported to undergo an excited-state charge transfer from the pyrrolic to the pyridyl moiety [25]. As a result, the emission of (7-aza)Trp is sensitive to the solvent polarity. The resulting solvatochromism effect leads to the red shift of emission from 350 nm in tetrahydrofuran to 402 nm in water. In the absence of **CaM**, upon 310 nm excitation where only (7-aza)Trp in (7-aza)Trp-**Baa** is excited, the emission of (7-aza)Trp-**Baa** maximized at ~ 400 nm which is nearly the same as that of (7-aza)Trp in the neutral Tris buffer, indicating that the photophysical property of (7-aza)Trp in (7-aza)Trp-**Baa** remains unchanged in comparison to that of the free (7-aza)Trp. Upon titrating (7-aza)Trp-**Baa** by **CaM**, (Fig. 1(a)), the original emission peak (400 nm) gradually blue shifted to 358 nm, accompanied by an increase in emission intensity, showing the change in polarity to a less polar environment around the (7-aza)Trp residue upon binding **CaM**.

Similar to that of (2,7-aza)Trp [1], multiple emission bands were observed for (2,7-aza)Trp-**Baa** in neutral Tris buffer, consisting of the N(1)-H isomer emission (340 nm), N(2)-H emission (380 nm) and N(7)-H emission (500 nm) (Fig. 1(b)), in which the population of the N(7)-H emission resulted from the water catalyzed proton transfer of the N(1)-H isomer (Scheme 1(b)).

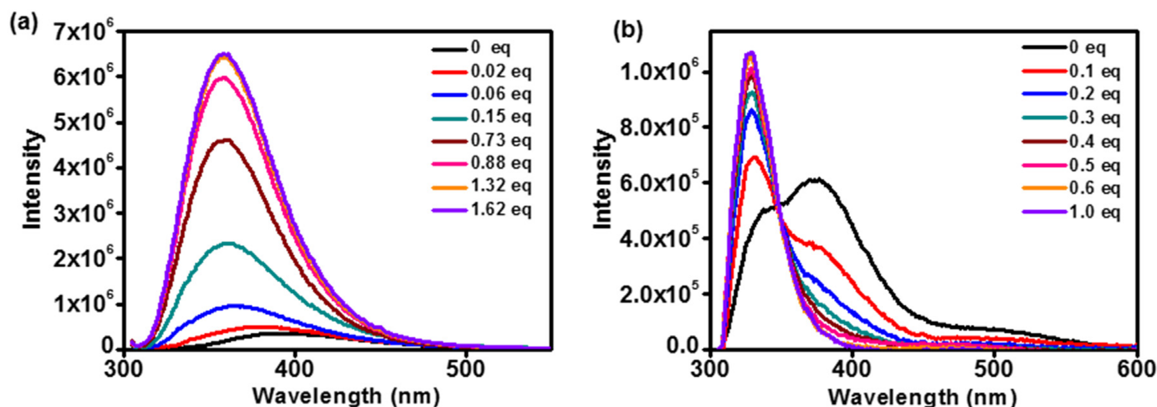

Fig. 1. The emission spectra of (a) (7-aza)Trp-**Baa** and (b) (2,7-aza)Trp-**Baa** in pH 7.5 Tris buffer with 1.0 mM $CaCl_2$ upon adding various amounts of **CaM**.

Dramatic changes in spectral features were observed during the titration of (2,7-aza)Trp-**Baa** by **CaM**, in which the N(2)-H (380 nm) and N(7)-H (500 nm) emission bands gradually decreased, accompanied by the increase of the N(1)-H emission at 340 nm. At the end of the titration, only the N(1)-H 340 nm emission was observed. The result of this steady-state titration can be interpreted by the following scenario. On the one hand, the (2,7-aza)Trp-**Baa/CaM** binding causes the loss of bulk water surrounding (2,7-aza)Trp-**Baa**, explaining the lack of the N(2)-H population (vide supra). On the other hand, the disappearance of the N(7)-H emission reflects the water scant environment surrounding (2,7-aza)Trp after forming the **CaM**/(2,7-aza)Trp-**Baa** complex, prohibiting the N(1)-H→N(7)-H proton transfer reaction in the excited state.

The corresponding emission relaxation dynamics further supports the aforementioned rationale. Fluorescence relaxation dynamics was used to monitor the N(1)-H emission in the absence **CaM** and presence of excess amount of **CaM**, (Fig. 2, Table 1). The 400 nm N(1)-H emission of (7-aza)Trp-**Baa** in neutral Tris buffer was measured to be 0.65 ns, which is the same as that (0.65 ns) of (7-aza)Trp in water (Table 1), indicating that (7-aza)Trp in (7-aza) Trp-**Baa** is fully exposed to water. The water catalyzed proton transfer takes place in the excited state, followed by the protonation of the tautomer and then fully quenching of the tautomer emission [26]. Upon binding **CaM,** the 358 nm N(1)-H emission lifetime of (7-aza)Trp-**Baa** was exceedingly long, measured to be as long as 7.35 ns. The result unambiguously concludes the lack of water molecules surrounding (7-aza)Trp upon **CaM** binding, resulting in the prohibition of the proton transfer reaction. In a qualitative manner, the lifetime of 7.35 ns is between that of 7-azaindole in acetonitrile (5.7 ns) and in DMSO (9.3 ns) [25], implying a less polar environment for (7-aza)Trp-**Baa** bound to

Table 1
Photophysical properties of various azaindole and azatryptophan analogues in neutral water and normal emission fluorescence lifetime of Trp replaced **Baa** in free peptide and **Baa-CaM** complexes.

	λ_{abs} (nm)	λ_{em} (nm)	λ_{mon} (nm)[a]	τ (ns)[b]
7-azaindole	288	386		0.91
(7-aza)Trp	289	400		0.65
2,7-diazaindole	295	335	320	0.22
		370	400	10.10
		495	550	0.21[rise], 1.30
(2,7-aza)Trp	300	340	320	0.26
		380	380	0.27(12%), 10.07 (88%)
		500	540	0.26[rise], 0.58
(7-aza)Trp-**Baa**	300	400	400	0.65
(7-aza)Trp-**Baa**+**CaM**	300	358	360	7.35
(2,7-aza)Trp-**Baa**	300	340	330	0.21
(2,7-aza)Trp-**Baa**+**CaM**	300	340	330	1.10

Values in parentheses indicate percentages and in brackets denote the rise component.

[a] The wavelength at which the measurement of relaxation dynamics was monitored.

[b] Values are ±0.03 ns in uncertainty.

CaM compared to free (7-aza)Trp-**Baa** in water, consistent with the spectral blue shift in the steady-state titration.

Free (2,7-aza)Trp-**Baa** in water was monitored at the N(1)-H emission at 330 nm (Fig. 2(b)). The lifetime of this emission was measured to be 0.21 ns; the quenching of which is mainly dominated by the water assisted proton transfer reaction in the excited state [1]. Upon binding **CaM**, the N(1)-H emission at 330 nm of

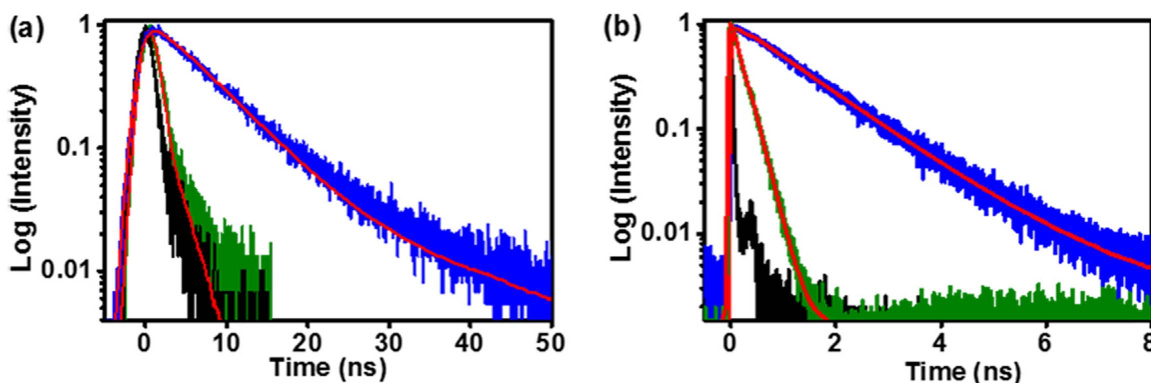

Fig. 2. The emission decay of (a) free (7-aza)Trp-**Baa** (green) and its binding with **CaM** (blue) monitored at 400 nm and 360 nm, respectively, and (b) free (2,7-aza)Trp-**Baa** (green) and its binding with **CaM** (blue) monitored at 330 nm. The instrument response and the fitting curves are marked in black and red lines, respectively.

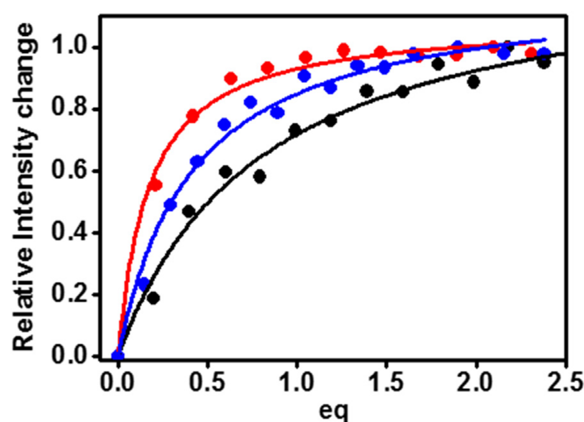

Fig. 3. The fluorescence titration plot of (2,7-aza)Trp-**Baa** (red), (7-aza)Trp-**Baa** (blue) and **Baa** (black) upon addition of **CaM**. The relative dissociation constant (k_d) was deduced to be 45 pM for (2,7-aza)Trp-**Baa**, 109 pM for (7-aza)Trp-**Baa** and 210 pM for **Baa** upon binding **CaM**.

(2,7-aza)Trp-**Baa** exhibited a much longer lifetime of 1.10 ns (Fig. 2(b)), which is similar to that (1.3 ns) of (2,7-aza)Trp65 in Thromboxane synthase [1]. The result firmly concludes the lack of water molecules surrounding (2,7-aza)Trp upon forming the (2,7-aza)Trp-**Baa**/**CaM** complex. In brief, the combination of steady-state spectra and the corresponding emission relaxation dynamics clearly indicates that the formation of **Baa**/**CaM**, ((7-aza)Trp-**Baa**/**CaM**, and (2,7-aza)Trp-**Baa**/**CaM**) complexes should squeeze out the water molecules at the interface, leading to a less polar and water scant, i.e. more hydrophobic, environment surrounding Trp.

We then further determined the affinities of (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa** for binding **CaM**. For the control experiment, we synthesized the same peptide sequence (**Baa**) reported by DeGrado et al. [27]. The **Baa**/**CaM** complex is in 1:1 stoichiometry and this relationship is also observed in our fluorescence titration experiment. DeGrado et al. used a competition method [28], in which **Baa** replaces melittin from the melittin-**CaM** (k_d=3 nM) bound resin to form the **Baa**-**CaM** complex, they determined the dissociation constant to be 210 ± 10 pM for **Baa** [28]. The titration of **Baa** (black), (7-aza)Trp-**Baa** (blue) and (2,7-aza)Trp-**Baa** with **CaM** was monitored by fluorescence at 320 nm, 358 nm and 380 nm, respectively (Fig. 3). Compared to that (210 ± 10 pM) of **Baa**/**CaM**, the k_d of (7-aza)Trp-**Baa**/**CaM** and (2,7-aza)Trp-**Baa**/**CaM** was then deduced to be 109 ± 5 pM and 45 ± 2 pM, respectively. Interestingly, replacing Trp with the unnatural (7-aza)Trp and (2,7-aza)Trp increases the binding affinity by 1.9 and 4.7 times, respectively. The results indicate that Trp may play a crucial role in **CaM** binding. This is reminiscent of a previous report on caldesmon, an actin-binding protein existing in both smooth muscle and nonmuscle cells, in which replacing Trp with alanine significantly reduced the **CaM** binding affinity [29]. In our case, replacing Trp with (7-aza)Trp or (2,7-aza)Trp resulted in increased **CaM** binding affinity instead. The result may not be too surprising since (7-aza)Trp and (2,7-aza)Trp share the same parent structure as Trp, so that the overall geometric shape is maintained. Nonetheless, the presence of the extra nitrogen heteroatom(s) in the indole ring may impose specific interaction such as hydrogen bonding with **CaM**. However, this notion remains to be confirmed due to the lack of structural information for the **Baa**/**CaM**, (7-aza)Trp-**Baa**/**CaM,** and (2,7-aza)Trp-**Baa**/**CaM** complexes.

4. Conclusions

In summary, we demonstrated the strategy of combining (7-aza)Trp and (2,7-aza)Trp to probe peptide-protein interactions, in which (7-aza)Trp is able to probe the polarity changes whereas (2,7-aza)Trp is sensitive to the water environment in both steady state emission and the corresponding relaxation dynamics. We applied peptides (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa**, in which Trp in **Baa** was replaced with the corresponding unnatural Trp analogue, to study the **Baa**/**CaM** binding process. The collective experimental results clearly indicate that the **Baa**/**CaM** ((7-aza)Trp-**Baa**/**CaM** and (2,7-aza)Trp-**Baa**/**CaM**) complexation removes the interface water molecules, leading to a less polar and water scant, i.e. more hydrophobic environment surrounding Trp. The **CaM** binding affinity for the peptides followed the trend **Baa**/**CaM** < (7-aza)Trp-**Baa**/**CaM** < (2,7-aza)Trp-**Baa**/**CaM**, which correlates well with the number of nitrogen atoms added in the indole moiety. Since the size and conformation are expected to be similar among **Baa**/**CaM**, (7-aza)Trp-**Baa** and (2,7-aza)Trp-**Baa**, the result may imply additional hydrogen bonding through N(7) or N(2) nitrogen atoms, or both, showing the role of Trp involved in the **Baa**/**CaM** interaction. These results demonstrate that (7-aza)Trp and (2,7-aza)Trp are complementary and serve as excellent probes for exploring the environment at the interface of protein-peptide interactions, which may prove to be valuable upon extending to protein-protein interaction. Also, it may be useful tools in the design of new peptides focusing on the development of bio-drugs.

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Transparency document. Supplementary material

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.05.022.

Appendix A. Supporting information

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