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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.

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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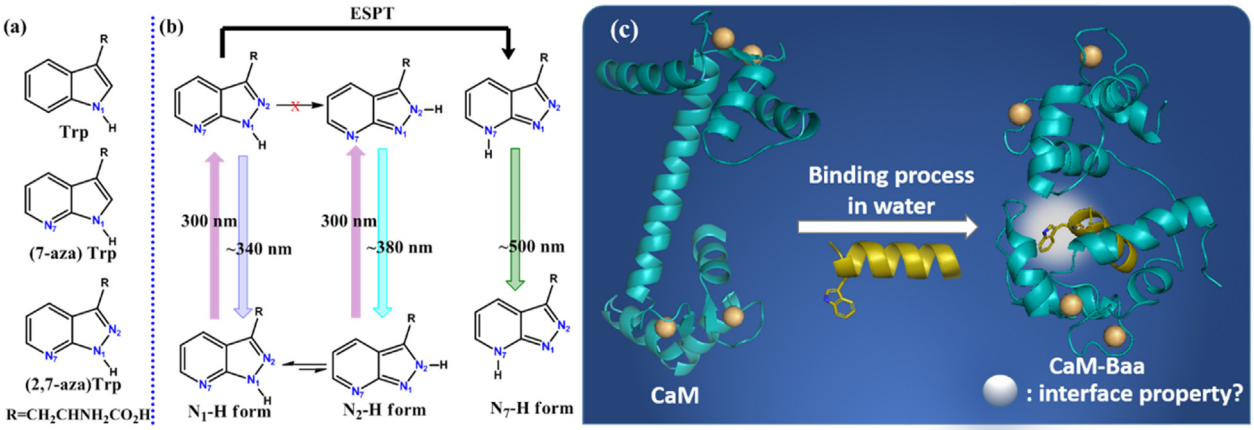

Scheme 1. (a) The chemical structures of Trp, (7-aza)Trp and (2,7-aza)Trp. (b) The ground-state equilibrium between N(1)-H and N(2)-H for (2,7-aza)Trp in neutral water and the water-catalyzed N(1)-H → N(7)-H proton transfer in the excited state for (2,7-aza)Trp. (c) Qualitative diagram of **CaM** and **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).