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Metal ions-induced conformational change of P_{23} by using TNS as fluorescence probe

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Abstract

In 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), pH 7.4, 25 °C, the conformational change of the truncated form of *ciliate Euplotes Octocarinatus* centrin (P_{23}) induced by metal ions were investigated using 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) as a probe. The results show that upon metal ions binding, P_{23} undergo a conformational change and the contributions to the conformational change from the two EF-hands are different, and Tb^{3+} has more larger influence than Ca^{2+} with the same concentration metal ions, which provide possible the evidence that the different EF-hands play distinct biological functions. Meanwhile, the conditional binding constants of TNS and Ca_2-loaded or Tb_2-loaded P_{23} were obtained, K (Ca_2-P_{23} + TNS) = $(7.49 \pm 0.88) \times 10^5$ mol^{-1} L, K (Tb_2-P_{23} + TNS) = $(8.24 \pm 0.49) \times 10^5$ mol^{-1} L.

Keywords: Conformation change; TNS; Metal ions

1. Introduction

Conformational changes are intrinsic to the function of a variety of proteins. Centrins are known to change conformation upon Ca^{2+} binding and often are termed Ca^{2+} sensor proteins, members of the EF-hand superfamily, and closely related to calmodulin, are part of the centrosome structure and are essential components of the centriole duplication process [1–3]. These proteins contain four helix–loop–helix domains, the so-called EF-hands, which may each bind one Ca^{2+} [4]. Like other proteins from the CaM subfamily, in their Ca-loaded form, centrins interact with specific target proteins to modulate their cellular activity [5]. The functions of centrins are actually poorly understood, up to now, centrins may play an additional role as components of Ca^{2+}-sensitive contractile fibers attached to centrosomes/basal bodies, as in the striated flagellar roots of green algae [6,7], the protein is required for proper cell division [8,9] and required for the normal duplication and separation of the microtubule-organizing center in their respective cells [10–12] and been shown to be essential for cell viability [13,14], and so on. C-terminal domain and N-terminal domain have been studied by several laboratories and it is an efficient method to understand better the function of centrins and the relation between function and amino acid sequence. TNS is a useful probe of conformational changes [15,16], because its fluorescence is altered when it binds to hydrophobic patches on the accessible surface of proteins.

Ciliate Euplotes Octocarinatus centrin was the first reported by our laboratory [17], which can coordinate four Ca^{2+} or Tb^{3+} ions in the same binding sites with two high affinity sites in C-terminal and two low affinity sites in N-terminal [18]. In this report, we got a truncated *Ciliate Euplotes Octocarinatus* centrin protein (P_{23}), including the second EF-hand domain in N-terminal and third EF-hand domain in C-terminal using the biological engineering method, and have studied the

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conformation change of P_{23} in the presence of metal ions (Ca^{2+} or Tb^{3+}) using the fluorscence probe TNS.

2. Materials and methods

2.1. Reagents

Hepes buffer, salts, and other chemicals utilized in protein purification were reagent grade. The purity of Tb_4O_7 is more than 99.9%, TNS was purchased from Sigma.

2.2. Stock solutions

A stock solution of terbium was prepared by dissolving weighed Tb_4O_7 in hydrochloric acid, which was standardized by complexiметric titration with EDTA using xylenol orange as indicator in HAc/NaAc buffer at pH 5.5. The solution of TNS prepared by dissolving weighed samples. The calcium stock solution was prepared by dissolving $CaCl_2$ in a small volume of distilled water. The solution was diluted into a certain volumetric flask with deionized water.

2.3. Protein expression and purification

Recombinant P_{23}, including two EF-hands domain, was expressed and purified as described [17]. A pair of primers, p1 (5′-GGC GGA TCC GGA TTT GAT GTT AAA AAG CC-3′) and p2 (5′-GCC GTC GAC TTA CTC ATC AGA TAA GTT-3′) with *BamH I* and *Sal I* restriction site were designed to obtain the expression recombinant plasmids pGEX-6P-1-P_{23}. The 76 residue construct used in this study consists of residues Glu62 through Glu137, with an additional Gly-Leu-Met-Leu sequence at the N terminus left over from PCR, the sequence of the clone was confirmed by commerical company. The protein concentration was measured spectrophotometrically using molar extinction coefficients at 280 nm of 2800 M^{-1} cm^{-1} for P_{23}. The extinction coefficient of centrin was estimated from the Tyr and Trp content as described by Pace et al. [19].

2.4. Fluorescence spectroscopy

All fluorescence experiments were performed on a Hitachi F-2500 at 25 °C, with all slit widths set to 10 nm. Excitation wavelength was 320 nm for TNS. Fluorescence emission spectra were recorded with a single scan over the range 350–600 nm for TNS. For the conformational characterizations by fluorescence, small aliquots of appropriate dilutions of a 548 μM $CaCl_2$ standard solution or 228 μM Tb^{3+} were added to a sample containing either 21 μM TNS plus 21 μM decalcified P_{23} in 10 mM Hepes, pH 7.4, 25 °C. For the TNS binding measurements, a control experiment was also performed, demonstrating that Ca^{2+} or Tb^{3+} alone has no effect on TNS fluorescence. Small aliquots of appropriate dilutions of a 127 μM TNS standard solution were added to a sample containing 21 μM Ca-loaded-P_{23} or Tb-loaded-P_{23} in 10 mM Hepes, pH 7.4, 25 °C.

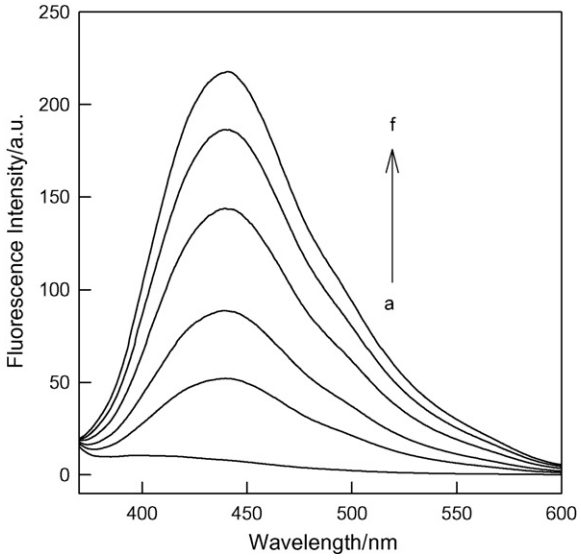

Fig. 1. Fluorescence spectra of Ca^{2+} binding to P_{23} in the presence of TNS. (a) [Ca]/[P_{23}] = 0, (b) [Ca]/[P_{23}] = 1, (c) [Ca]/[P_{23}] = 1.4, (d) [Ca]/[P_{23}] = 1.6, (e) [Ca]/[P_{23}] = 1.8, (f) [Ca]/[P_{23}] = 2.0. The sample contains 21 μM P_{23} plus 21 μM TNS.

3. Results and discussion

3.1. The conformation change of P_{23}

A set of fluorescence spectra caused by the addition of aliquots of Ca^{2+} (548 μM) solution to 2 mL P_{23} (21 μM) in the presence of 21 μM TNS in 10 mM Hepes at pH 7.4, 25 °C is shown in Fig. 1. The fluorescence of TNS binding the Ca_2-loaded P_{23} or Tb_2-loaded P_{23} can be seen in Fig. 2 and the plot of F_{440}/[P_{23}], the fluorescence intensity of TNS at 440 nm divided by the concentration of P_{23}, *versus* [Tb^{3+}]/[P_{23}] is shown as Fig. 3.

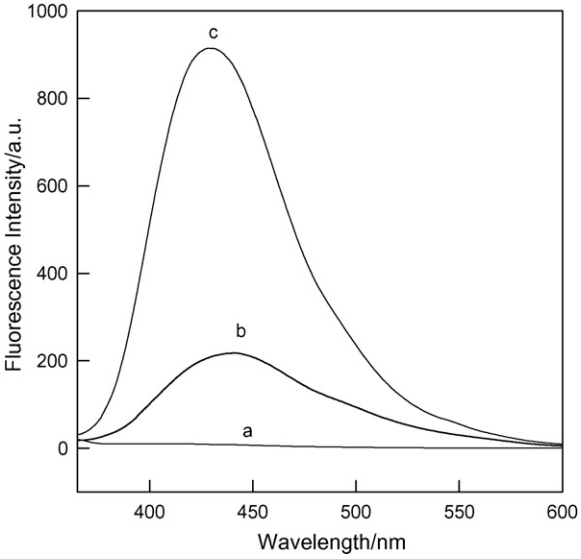

Fig. 2. Fluorescence spectra of TNS in the presence of Tb_2-loaded P_{23} or Ca_2-loaed P_{23}. (a) TNS in 10 mM Hepes, (b) in the presence of Ca_2-loaded P_{23} and (c) in the presence of Tb_2-loaded P_{23}. The concentration of TNS is 21 μM.

It can be seen from Fig. 1 that there is a peak at near 440 nm, and which increase obviously with the addition of metal ions, when the value of [metal ions]/[P_{23}] is equal to near 2, the increase become very slow (Fig. 3). The increase is owing to the fluorescence change of TNS in different hydrophobic environment, TNS is one of a class of compounds which do not fluoresce in water but fluoresce both in organic solvents or hydrophobic environment [15]. From the Fig. 1, the results show that P_{23} undergo a conformational change when binding the Ca^{2+}, and the change extent of hydrophobic exposure between the second EF-hand and the third EF-hand is different and the second EF-hand domain is larger than the third EF-hand domain, induced by metal ions (Fig. 3). Undergoing a large conformational is required for the trigger proteins (Ca^{2+}-modulated proteins or sensor proteins such as CaM and troponin C) to regulate a vast number of target proteins [20–22]. So the second EF-hand domain of N-terminal and the third EF-hand domain of C-terminal play a distinct role in the process of centrin realizing itself biological function. This is a possible explanation of that N-terminal of centrin responsible for self-assembly, while C-terminal serve as recognizing the target protein or enzyme, which is accord with the reports previously [23,24].

Comparison the curve b with c of Fig. 2, Tb^{3+} has a more larger influence to the conformation of P_{23} than Ca^{2+}, owing to higher positive charge (+3).

3.2. Binding constants

3.2.1. Binding of TNS to Tb-loaded P_{23}

From the experiments above, it can be seen that near [metal ions]/[P_{23}] = 2, the fluorescence intensity of TNS increase weakly, suggesting that TNS bind the hydrophobic paths on the accessible surface of protein, which is mostly from the hydrophobic surface exposure induced by binding metal ions.

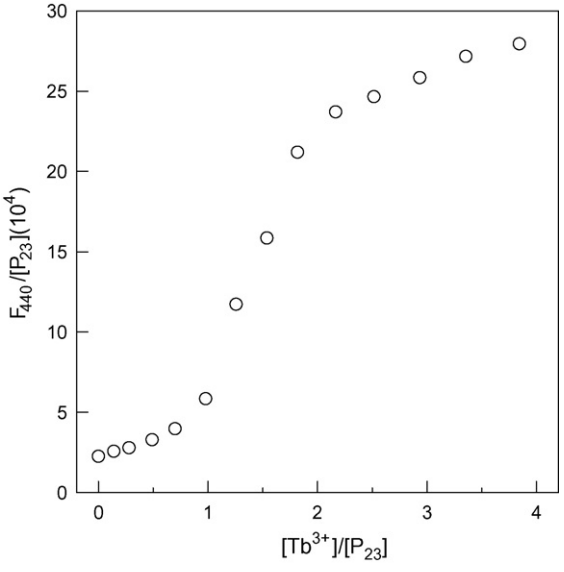

Fig. 3. Titration curve of the addition Tb^{3+} to the P_{23} in the presence of TNS, in 10 mM Hepes, pH 7.4, 25 °C. The sample contains 21 μM P_{23} and 21 μM TNS.

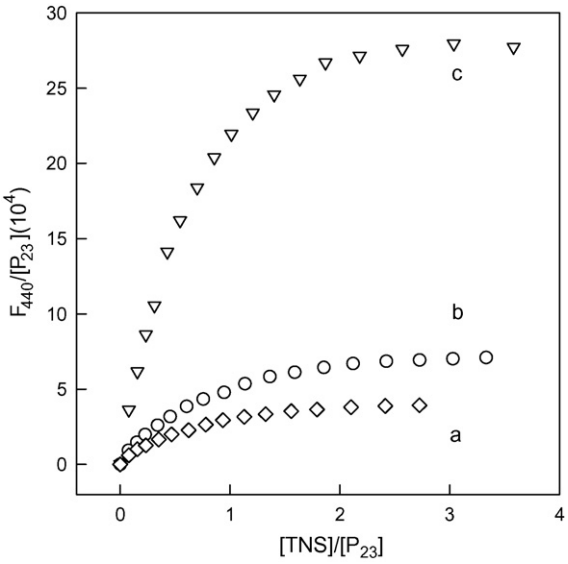

Fig. 4. Titration curve of the addition TNS to the different form of P_{23}, in 10 mM Hepes, pH 7.4, 25 °C. (a) apoP_{23}, b: Ca_2-loaded P_{23}, (c) Tb_2-loaded P_{23}. The concentration of P_{23} is 21 μM.

So, we can calculate the conditional binding constants of TNS and the different form of P_{23} (Fig. 4). Curve c in Fig. 4 is the fluorescence titration curve of TNS to Tb_2-loaded P_{23}. Assuming that there are n TNS-binding sites and they are independent and identical in Tb_2-loaded P_{23}, the conditional binding constant [18,25] can be fitted using Eqs. (1)–(7) from the data of curve c in Fig. 4.

$$n\mathrm{TNS} + \mathrm{P} \leftrightarrow \mathrm{TNS}_n\mathrm{P} \tag{1}$$

$$F_\infty \propto n[\mathrm{P}]_\mathrm{t} \tag{2}$$

$$F_\mathrm{r} \propto [\mathrm{TNS}]_\mathrm{b} \tag{3}$$

$$[\mathrm{TNS}]_\mathrm{b} = \frac{F_\mathrm{r}}{F_\infty} n[\mathrm{P}]_\mathrm{t} \tag{4}$$

$$K = \frac{[\mathrm{TNS}]_\mathrm{b}}{[\mathrm{TNS}]_\mathrm{f} \cdot [\mathrm{P}]_\mathrm{f}} \tag{5}$$

$$\frac{[\mathrm{TNS}]_\mathrm{b}}{[\mathrm{TNS}]_\mathrm{t} - [\mathrm{TNS}]_\mathrm{b}} = K\{n[\mathrm{P}]_\mathrm{t} - [\mathrm{TNS}]_\mathrm{b}\} \tag{6}$$

$$\frac{[\mathrm{TNS}]_\mathrm{t}}{[\mathrm{TNS}]_\mathrm{b}} = 1 + \frac{1}{K\{n[\mathrm{P}]_\mathrm{t} - [\mathrm{TNS}]_\mathrm{b}\}} \tag{7}$$

where $[\mathrm{TNS}]_\mathrm{t}$, $[\mathrm{TNS}]_\mathrm{b}$ is the total, bound concentration of TNS, respectively. $[\mathrm{P}]_\mathrm{t}$, $[\mathrm{P}]_\mathrm{f}$ is the total or free concentration of Tb_2-loaded P_{23}. In 10 mM Hepes, pH 7.4, 25 °C, K (Tb_2-P_{23} + TNS) can be calculated to be $(8.24 \pm 0.49) \times 10^5$ mol^{-1} L, and the number of binding sites is 1 for Tb_2-loaded P_{23} from Fig. 5.

3.2.2. Binding of TNS to Ca_2-loaded P_{23}

Using the TNS titrated the Ca_2-loaded P_{23}, the likely results can be seen shown in Fig. 4 curve b. In the similar way, we can obtained that in 10 mM Hepes, pH 7.4, 25 °C, K (Ca_2-P_{23} + TNS) = $(7.49 \pm 0.88) \times 10^5$ mol^{-1} L.

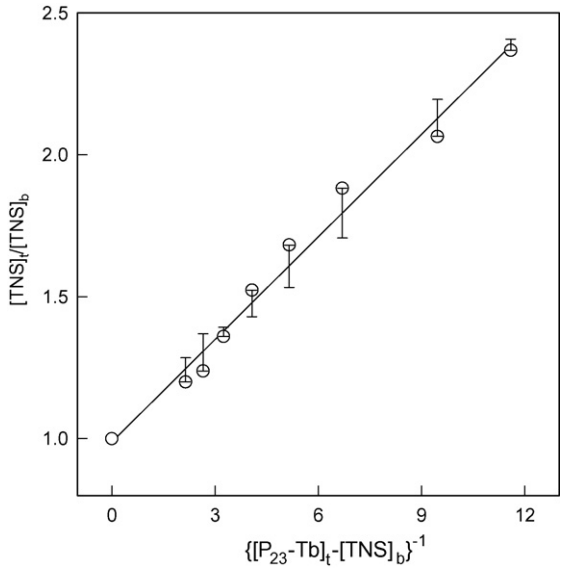

Fig. 5. The plot of $[TNS]_t/[TNS]_b$ *vs.* $\{[P_{23}\text{-}Tb_2]_t\text{-}[TNS]_b\}^{-1}$.

It can be seen from Fig. 4 that affinity of TNS binding to different form P_{23} is different, Tb_2-P_{23}-TNS > Ca_2-P_{23}-TNS > apoP_{23}-TNS, showing that the Tb^{3+} infulence the conformation of protein in a larger than Ca^{2+} extent, if rare earth ions access to the cell, which will substitute Ca^{2+} and arise the corresponding the change to protein. These changes can be able to affect the many functions of the cell and possibly play a promote role in the corps grow.

4. Conclusions

Upon the metal ions (Ca^{2+} or Tb^{3+}), P_{23} undergo a different extent conformation change, Tb^{3+} has been shown having much larger extent change than Ca^{2+} when bind to P_{23}, as shown in Figs. 1 and 2, and then the second EF-hand domain and the third EF-hand domain have the different affinity to Tb^{3+}, meanwhile, whose conformation change is different with binding the same equivalents metal ions, indicating that their biological function is different. In the end, the binding constants were obtained, K (Ca_2-P_{23} + TNS) = $(7.49 \pm 0.88) \times 10^5 mol^{-1}$ L, K (Tb_2-P_{23} + TNS) = $(8.24 \pm 0.49) \times 10^5$ mol^{-1} L.

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