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Analysis of Tb^{3+}- and melittin-binding with the C-terminal domain of centrin in *Euplotes octocarinatus*

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ABSTRACT

Centrin is a low molecular mass (20 KDa) protein that belongs to the EF-hand superfamily. In this work, the interaction between the Tb^{3+}-saturated C-terminal domain of *Euplotes octocarinatus* centrin (Tb_2-C-EoCen) and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) was investigated using difference UV–vis spectra and the fluorescence spectra methods. In 100 mM N-2-hydroxy-ethylpiperazine-N-2-ethane-sulfonic acid (Hepes) at pH 7.4, with the addition of Tb_2-C-EoCen, four new peaks were observed at 265 nm, 278 nm, 317 nm and 360 nm by absorptivity compared with blank solution of TNS. At the same time, the reaction could be measured by fluorescence spectra. The fluorescence emission of TNS was shifted from 480 nm to 445 nm in the presence of Tb_2-C-EoCen. Meanwhile, its fluorescence intensity was increased markedly. The 1:1 stoichiometric ratio of C-EoCen to TNS was confirmed by fluorescence titration curves. The conditional binding constants of TNS with C-EoCen and Tb_2-C-EoCen were calculated to be log $K_{(C\text{-}EoCen\text{-}TNS)}=5.32\pm0.04$ M^{-1} and log $K_{(Tb2\text{-}C\text{-}EoCen\text{-}TNS)}=5.58\pm0.12$ M^{-1}, respectively. In addition, the protein of Tb_2-C-EoCen binding with melittin was also studied. Based on the fluorescence titration curves, the 1:1 stoichiometric ratio of Tb_2-C-EoCen to melittin was confirmed. And the conditional binding constant of C-EoCen with melittin was calculated to be log $Ka'=6.79\pm0.17$ M^{-1}.

1. Introduction

The centrosome is an exclusive structure found in eukaryotic cells, also known as the microtubule-organizing centers (MTOC). It consists of about 100 proteins one of which is centrin [1–3]. Centrin plays fundamental roles in many cellular processes, including chromosomal segregation, cytokinesis, fertilization, cellular morphogenesis, cell motility, and intracellular trafficking [4]. Abnormal centrosome duplication may lead to chromosomal instability and cancer, which was supported by discovery of supernumerary abnormal centrosomes in different human tumor [5–7]. In addition, centrin forms part of the human heterotrimetric DNA damage recognition complex required for global genome nucleotide excision repair [8]. Centrins, also termed "caltractin", are highly conserved low molecular weight proteins belonging to the superfamily of EF-hand Ca^{2+}-binding proteins [4,9]. The first centrin was discovered as the major component of striated flagellar rootlets associated with the basal bodies of unicellular green algae [10]. Structurally, one centrin molecule contains four helix-loop-helix motifs called EF-hands, one pair of which is capable of binding two Ca^{2+} [6]. The binding of Ca^{2+} may involve structural rearrangement of α-helices with the consequent exposure of hydrophobic cleft, often referred as the "Ca^{2+}" switch [7].

Lanthanides ions (Ln) have been known for their diversity in biological effects, and the application of Ln in medicine has high potential. In agriculture, it has been used to increase the production of crops and to promote the growth of livestock in China for many years [9]. However, the molecular mechanism of the biological effects of Ln remains not completely understood so far. Ln has similar ionic radii and similar coordination properties to Ca^{2+} [10]. Hence, Tb^{3+} was usually used to sense properties of Ca^{2+}-binding proteins [11].

Ciliate Euplotes octocarinatus centrin (EoCen) is the first reported by our laboratory (gene register Y18899) [12], which is cloned from *Euplotes octocarinatus*, and the detailed biological function is unclear. It is a protein of 168 residues, which shares about 60, 62 and 66% sequence identity with human centrin 1 (HsCen1), human centrin 2 (HsCen2) and human centrin 3 (HsCen3), respectively. It shares approximately 50% sequence identity with the well studied EF-hand protein calmodulin

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(CaM). Like CaM, EoCen 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 [13]. In this paper, the C-terminal domain of *Euplotes octocarinatus* centrin (C-EoCen, 90–168 aa) was purified as described previously [12,14]. Using TNS as fluorescence probe, Tb^{3+} binding with C-EoCen was investigated by the spectral methods. C-EoCen undergoes a conformational change induced by Tb^{3+} binding from "closed" to "open" state. For investigating the subsequent effect of metal ions-induced conformational change, interaction of Tb^{3+}-saturated C-EoCen with melittin was monitored in 100 mM Hepes and 150 mM NaCl at pH 7.4. Results suggested that melittin binds with Tb_2-C-EoCen at the ratio of 1:1. And the conditional binding constant between them was also calculated.

2. Materials and methods

2.1. Reagents

N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes), 2-*p*-toluidinylnaphthalene-6- sulfonate (TNS) and melittin were purchased from Sigma. Hepes, TNS, salts and other chemicals utilized in protein purification are of analytical grade. Disodium ethylenediaminetertracetic acid (EDTA) and calcium chloride ($CaCl_2$) were purchased from Shanghai in China.

A solution of Tb^{3+} 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 was prepared by dissolving weighed samples.

2.2. Protein preparation

The C-terminal domain of *Euplotes octocarinatus* centrin (C-EoCen) was expressed and purified as described previously [15]. Using genes of EoCen as template, C-EoCen was obtained by PCR through primers of 5′-CCGGGATCCATTGGATTTGATGATTTTCTTGATATTATG-3′ and 5′-CCGACGTCGACAGTGATTGCCA- AGC-3′. Then it was subcloned into expression vector of pGEX-6p-1. The recombinant proteins of C-EoCen on vector of pGEX-6p-1 were constructed. After verification by DNA sequence analysis, the recombinant plasmid was transferred into *E. coil* (DE3), which was incubated at 37 °C. At an optical density of 0.6–0.8 (at 600 nm), protein synthesis was induced using IPTG (0.5 mM) for 3.5 h. GST-C-EoCen was purified as a GST fusion protein using glutathione sepharose 4FF in PBS buffer (in mM): KH_2PO_4 1.8, Na_2HPO_4 10 and KCl 2.7 (pH 7.4). The GST fusion proteins were then cleaved by PreScission Protease (PPase).

The purity of the intermediate and final samples was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After purification, the proteins were concentrated and kept at −80 °C. The stock protein solutions were conserved in 100 mM Hepes.

The concentration of protein C-EoCen was measured by its absorption at 280 nm with an extinction coefficient of $\varepsilon_{280}=1400\ M^{-1}\ cm^{-1}$.

The concentration of melittin, the 26-residue peptide (GIGAVLKVLTTGLPALISWIKRKR QQ), was determined from A_{280} measurements using molar extinction coefficient of $5500\ M^{-1}\ cm^{-1}$ [16,17].

2.3. Metal removal

To remove contaminating bound cations, the protein samples were first pretreated with EDTA and then passed through a 10 cm × 1 cm Sephadex G-25 column equilibrated in Hepes buffer (100 mM), at pH 7.4.

2.4. Difference UV–vis spectroscopy

Difference UV–vis spectra were recorded with a Hewlett Packard 8453 spectrophotometer. A mixed solution of 30 μL TNS (4.207×10^{-5} M) and 970 μL Hepes buffer (100 mM, pH 7.4) was added to a 1 cm sample cuvette, and then Tb_2-C-EoCen (1.96×10^{-5} M) solution was added to the above solution gradually to determine a value of the absorptivity of the TNS/Tb_2-C-EoCen. For C-EoCen binding with melittin, samples were prepared by gradually adding C-EoCen to the melittin solution in the presence of 1.0 mM $CaCl_2$.

An equilibrium time of 5 min was used between each titration. During the titration, the sample was maintained at 25 °C using a jacketed cell holder connected to an external circulating water bath (Huber).

2.5. Fluorescence spectroscopy

The Tb^{3+}-induced changes on C-EoCen were followed by monitoring fluorescence properties of TNS as described previously on Hitachi F-2500 [15]. The Tb^{3+} titrations were carried out in the presence of TNS on 20 μM metal-free C-EoCen excited with excitation at 320 nm in 100 mM Hepes and 150 mM NaCl at pH 7.4, 25 °C. A filter with a long pass of 300 nm was used to avoid secondary Raleigh scattering. The binding C-EoCen with melittin was carried out with excitation at 295 nm in 1.0 mM $CaCl_2$, 100 mM Hepes and 150 mM NaCl at pH 7.4, 25 °C. The slit widths for excitation and emission were 5 nm. An equilibrium time of 5 min was used between each titration. Fluorescence emission spectra were recorded with a single scan over the range 350–600 nm for TNS and 298–440 nm for melittin. For the TNS binding measurements, a control experiment was also performed, demonstrating that 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 20 μM metal-free C-EoCen and Tb_2-loaded C-EoCen, respectively, in 100 mM Hepes and 150 mM NaCl, pH 7.4, 25 °C.

3. Results and discussion

3.1. Expressing and purification of recombinant C-EoCen

The BL21 cell containing the plasmid of recombinant C-terminal domain of *Euplotes octocarinatus* (C-EoCen, 90-168 aa) was cultured at 37 °C for 3–4 h and induced by isopropyl-β-D-thiogalactopyranoside (IPTG 0.8 mM) for 3.0 h at an optical density of 0.6–0.8 (at 600 nm). The GST fusion proteins (GST-C-EoCen) were purified using glutathione Sepharose 4FF in PBS buffer. The GST fusion proteins were then cleaved by PreScission Protease (PPase) and GST tag was removed. The target protein C-EoCen was purified with about 35% yield (Fig. 1).

3.2. Difference UV–vis assays

Using TNS as blank solution, the difference UV–vis spectra of TNS in the absence and presence of Tb^{3+}-saturated C-EoCen were shown (Fig. 2). It is obvious that four peaks appeared at 265 nm, 278 nm, 317 nm and 360 nm, while Tb^{3+}-saturated C-EoCen was added into the solution of TNS. The absorbance at 278 nm enhanced, which resulted from the increase of the concentration of Tb_2-C-EoCen in the system. The absorbance of negative peaks at 265 nm, 317 nm and 360 nm decreased with the increasing of