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Characterization of melittin binding to *Euplotes octocarinatus* centrin

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Abstract

In the presence of 1.0 mM Ca^{2+}, the interaction between *Euplotes octocarinatus* centrin (EoCen) and melittin (ME) was studied by means of fluorescence spectra. In 0.1 M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) and 150 mM NaCl at pH 7.4, fluorescence peak of ME was observed at about 353 nm indicating that micro-environment of Tryptophan (Trp) residue in ME was hydrophilic. With the addition of 3.2×10^{-4} M calcium saturated EoCen (holoEoCen), the peak of ME was blue-shifted to 339 nm, which may be resulted from micro-environmental changes of the peptide. At the same time, fluorescence emission of ME was increased significantly suggesting that new complex of ME−holoEoCen was formed under the experimental conditions. Based on the fluorescence titration curves, the 1:1 stoichiometric ratio of holoEoCen to ME was confirmed. In addition, the conditional binding constant of holoEoCen with ME was calculated to be $\log K_{\mathrm{ME-holoEoCen}} = 6.59 \pm 0.14$.

Keywords: Centrin; Melittin; Fluorescence

1. Introduction

Centrin is an acidic, small size (∼20 KDa) highly conserved calcium-binding protein. It belongs to the calmodulin (CaM) superfamily and is closely related to CaM. Initially, centrins were identified in the unicellular green algae, such as *Tetraselmis striata* [1] and *Chlamydomonas reinhardtii* [2], as major components of Ca^{2+}-sensitive contractile fibers. Subsequently, they were identified from protozoa and yeast to plants and humans [3,4]. Due to high homology [5] and identical domain architecture, the tertiary structure of centrins may be similar to that of CaM. Like CaM, centrin is also comprised of two structurally independent globular domains connected by a flexible linker and each domain contains two helix–loop–helix calcium-binding motifs, so called EF-hands. Genetic studies show that centrin is essential to normal cell cycle-dependent duplication and segregation of the microtubule organizing center (MTOC) [6]. And centrin forms part of the human heterotrimetric DNA damage recognition complex required for global genome nucleotide excision repair [7]. However, studies about target peptides were little and mechanism of peptide binding with centrin is unknown. Kar1p was the subject of more detailed biophysical studies, which is unstructured in the absence of centrin [8]. Recently, another centrin target peptide HsXPC derived from human xeroderma pigmentosum group C protein was identified in the Human centrin 2 (HsCen2) binding site [9]. Papers about reactions of EoCen with probe TNS have been reported. Some characterizations including conformational changes of EoCen have been studied by our groups [10–12]. However, little data about properties of melittin (ME) binding to *Euplotes octocarinatus* centrin (EoCen) are presently available. EoCen is an acidic EF-hand protein, which was identified firstly by our labs (GenBank accession number: Y18899) (Fig. 1). In the present paper, the interaction between calcium saturated EoCen (holoEoCen) and ME was investigated in 0.1 M Hepes and 150 mM NaCl by means of fluorescence spectra. Results suggested that ME binds to holoEoCen at the ratio of 1:1. And conditional binding constant between them was also calculated.

2. Materials and methods

2.1. Materials

Melittin (ME) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) were reagent grade and got from

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MIKKPEFGLMQPP

E-helix Ca^{2+}-loop F-helix

28.....EF-handI.....56

KKRVRQELSEEQKQ**EIKEAFDLFDTNKTGSIDYHELKVAMRAL**

64.....EF-handII.....92

GFDVKKP**EILELMNEYDREGNGYIGFDDFLDIMTEK**

101.....EF-hand III.....129

IKNRDPVE**EILKAFKVFDEDNSGKISLRNLKRVAKELG**

137..... EF-hand IV.....165

ENLSDD**ELQAMIDEFDKDQDGEISEQEFLNIMKQTSIY**

Fig. 1. Amino acid sequence of *Euplotes octocarinatus* centrin (GenBank accession number: Y18899). The four EF-hand motifs are in bold and the fluorescent residues are underlined.

Sigma Ltd. Disodium ethylenediaminetertracetic acid (EDTA) and calcium chloride ($CaCl_2$) were purchased from Shanghai in China.

Potassium phosphate (KH_2PO_4, 1.8 mM), sodium phosphate (Na_2HPO_4, 10 mM), potassium chloride (KCl, 2.7 mM) and sodium chloride (NaCl, 140 mM) (PBS) were analytical reagents and they were purchased from Sangon in Shanghai.

The instruments are F-2500 fluorescence spectrophotometer, Hitachi 850 fluorescence spectrophotometer, and pH meter.

2.2. Methods

(i) Protein expression and preparation

ME, the 26-residue peptide (GIGAVLKVLTTGLPALISWIKRKRQQ) was purified as described previously [13]. Its concentration was determined from A_{280} measurements using molar extinction coefficient of 5500 M^{-1} cm^{-1} [14].

Recombinant EoCen was expressed and purified as described previously [15].

Protein samples were pretreated with EDTA and then passed through 60 cm × 1 cm Sephadex S 200 column equilibrated with Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffers.

(ii) Spectral measurements

Fluorescence spectra were recorded on an F-2500 fluorescence spectrophotometer. Fluorescence intensity was measured with a Hitachi 850 fluorescence spectrophotometer and excitation wavelength was at 295 nm. The slit widths for excitation and emission were 10 nm. A filter with a long pass of >310 nm was used to avoid secondary Raleigh scattering.

3. Results and discussion

Solution of ME was added to a dry fluorescence cuvette in 0.1 M Hepes and 150 mM NaCl at pH 7.4 and room temperature. Target peptide of ME (GIGAVLKVLTTGLPALISWIKRKRQQ) contains only one tryptophane (Trp) residue and EoCen contains only tyrosine (Tyr) residues. Therefore, the peptide can be selectively observed by excitation at 295 nm. In 0.1 M Hepes and 150 mM NaCl at pH 7.4, fluorescence emission of ME appeared near 353 nm, shown in Fig. 2b. With the addition of sequential aliquots of holoEoCen (3.2×10^{-4} M), fluorescence emission of ME was blue-shifted to 339 nm (Fig. 2c), indicating new complex has been formed under the experimental conditions. At the same time, fluorescence intensity of ME was increased significantly. As blank experiment, fluorescence emission of holoEoCen was monitored under same experimental conditions (Fig. 2a). Trp has been used widely to probe microenvironmental changes around it by the method of fluorescence spectra [16]. In hydrophilic environments, fluorescence emission of Trp located at about 350 nm and it will be shifted to about 320 nm while its environments are changed to hydrophobic environments. In the reactions of ME with holoEoCen, Trp in peptide may be buried completely in the deepest pocket on the holoEoCen surface. With the addition of holoEoCen, the conformation of ME was changed significantly. Hydrophobic interactions of interfaces between holoEoCen and peptides may be the major reasons resulting in stronger affinity. In addition, electrostatic interactions between highly basic peptide ME and the acidic holoEoCen as well as potential salt-bridges from peptide-centrin further complemented interactions of holoEoCen to ME.

To correct the dilution effect, fluorescence intensity at 339 nm was converted to molar fluorescence intensity (F_M) via dividing fluorescence intensity by analytical concentration of ME. The plot of F_M against r (r = [holoEoCen]/[ME]) was displayed in Fig. 3a. It can be seen that one break appeared at about $r = 1$, confirming the 1:1 stoichiometric ratio of ME−holoEoCen complex. As [holoEoCen]/[ME] < 1, the molar fluorescence intensity (F_M) of ME−holoEoCen was calculated to be $(4.40 \pm 0.01) \times 10^6$ M^{-1}. ME titrating holoEoCen further confirmed that ME binds to holoEoCen at the ratio of 1:1 (Fig. 3b). To determine the binding constant between holoEoCen and ME, plot of $[Melittin]_t/[Melittin]_b$ versus $1/([EoCen]_t - [Melittin]_b)$ was plotted in Fig. 4.

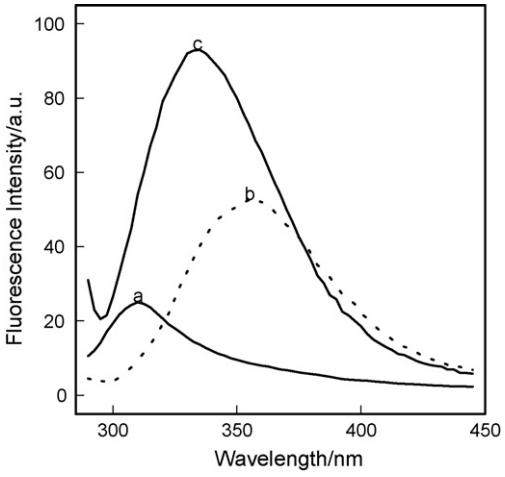

Fig. 2. HoloEoCen−ME interaction measured by Trp fluorescence of ME in 0.1 M Hepes and 150 mM NaCl, pH 7.4. Slits are 10 nm. Excitation is at 295 nm. Samples contain 5.17×10^{-5} M of the individual compounds or of the equimolar mixtures. holoEoCen (a), ME (b) and holoEoCen−ME (c).