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# Binding of *Euplotes Octocarinatus* centrin with target peptide melittin

ZHAO YaQin[1], FENG JiuYing[1], LIANG AiHua[2] & YANG BinSheng[1†]

[1] Institute of Molecular Science, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi University, Taiyuan 030006, China;
[2] Institute of Biotechnology, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi University, Taiyuan 030006, China

**Interactions between model target peptide melittin (ME) and *Euplotes octocarinatus* centrin (EoCen) were investigated by fluorescence spectra, circular dichroism (CD) spectra and native polyacrylamide gel electrophoresis (PAGE). In 0.1 mol/L N-2-hydroxyethylpiperazine-N-2-ethanesufonic acid (Hepes) and 150 mmol/L NaCl at pH 7.4, EoCen and isolated short C-terminal domain of EoCen (SC-EoCen) form 1∶1 peptide∶protein complexes. However, no detectable signal changes can be observed while isolated N-terminal domain of EoCen (N-EoCen) or isolated long C-terminal domain of EoCen (LC-EoCen) was added into solution of ME. The interaction between EoCen and ME is specified exclusively for the short C-terminal domain of EoCen. On the basis of fluorescence titration curves, the conditional binding constants of ME with EoCen and SC-EoCen were calculated to be log$K_{\text{ME-EoCen}}$ = 6.81±0.33 and log-$K_{\text{ME-SC-EoCen}}$ = 6.51±0.45, respectively.**

centrin, melittin, spectra

Centrin is closely related to the ubiquitous archetypal EF-hand calcium sensor protein calmodulin (CaM), which was highly conserved in diverse evolutionary lineages, including algal, higher plant, invertebrates, and mammalian cells[1,2]. As major components of $Ca^{2+}$-sensitive contractile fibers, they were initially identified in the unicellular green algae[3,4]. *Euplotes octocarinatus* centrin (EoCen) was an acidic low molecular mass protein, which was firstly cloned by the lab (GenBank accession number: Y18899). It consists of 168 residues, which share high sequence identity (50%) with CaM. Like CaM, centrins belong to EF-hand calcium-binding protein, which consists of two independent globular domains connected by a flexible linker. And each domain contains two helix-loop-helix calcium-binding motifs known as EF-hands.

Despite high sequence similarity, centrin recognizes target proteins distinct from those that partner with CaMs and other EF-hand family members. In contrast to CaM, the number of cellular targets binding with centrin remains small and little is known about structural basis.

Melittin (ME), the amphiphilic peptide from bee venom, is considered as good candidate for detailed structural studies[5]. It was also used as natural peptide to mimic CaM target peptides[6,7]. So far, no data on the ME binding properties of EoCen are presently available. ME-binding characteristics, i.e. affinity, selectivity, as well as the effect of ME on the conformation of EoCen were investigated by fluorescence spectra, circular dichroism (CD) and native polyacrylamide gel electrophoresis (PAGE).

## 1 Materials and methods

### 1.1 Materials

Melittin (ME) and N-2-hydroxyethylpiperazine-N-2-

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†Corresponding author (email: yangbs@sxu.edu.cn)
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ethanesulfonic acid (Hepes) were of reagents grade and got from Sigma Ltd. Disodium ethylenediaminetertracetic acid (EDTA) and calcium chloride ($CaCl_2$) were purchased from Shanghai, China.

Potassium phosphate ($KH_2PO_4$, 1.8 mmol/L), sodium phosphate ($Na_2HPO_4$, 10 mmol/L), potassium chloride (KCl, 2.7 mmol/L) and sodium chloride (NaCl, 140 mmol/L) (PBS) were analytical reagents and purchased from Sangon, Shanghai.

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

### 1.2 Methods

1.2.1 Protein expression and preparation. In the present work, three constructs of EoCen were used, namely, isolated N-terminal EoCen (N-EoCen 1M-101E) and two C-terminal fragments of EoCen: SC-EoCen (90T-168Y) covering two EF-hands and LC-EoCen (80I-168Y), having 10 additional residues.

The DNA of EoCen fragments was obtained by polymerization chain reaction from DNA of EoCen and subcloned into pGEX-6P-1. Recombinants N-EoCen, SC-EoCen and LC-EoCen were expressed and purified as described previously[8].

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

1.2.2 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 295 nm, slits were 5 nm, and fliter was 310 nm. A solution of ME in 100 mmol/L Hepes and 150 mmol/L sodium chloride at pH 7.4 was added to a dry fluorescence cuvette and titrated with protein.

For anisotropy measurements, exciting light at 295 nm was vertically polarized and passed. The Hitachi 850 fluorescence spectrophotometer was operated in the "L" format. Anisotropies were calculated directly from the following equations:

$$G = \frac{S_V}{S_H}, \quad (1)$$

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}, \quad (2)$$

where $I_{HV}$ corresponds to horizontally polarized excitation and vertically polarized emission. $I_{VV}$ represents vertically polarized excitation and emission. In addition, $S_V$ and $S_H$ are sensitivity of the emission channel for the vertically and horizontally polarized components, respectively.

Circular dichroism (CD) experiments were performed on Jasco J-810 spectropolarimeter equipped with Peltier temperature controller. Far-UV spectra were recorded between 195 and 250 nm using 1 mm quartz cells. Samples were dissolved in 10 mmol/L Hepes and 150 mmol/L NaCl at pH 7.4. The appreciative α-helix content of protein ($f_h$) was evaluated using the equation provided by Chen and Yang [7]

$$f_h = -\frac{|\theta|_{222} + 2340}{30300}. \quad (3)$$

Samples of ME in 10 mmol/L Hepes and 150 mmol/L NaCl at pH 7.4 was added to a dry fluorescence cuvette and titrated with EoCen. An equilibrium time of 5 min was used for each titration.

## 2 Results and discussion

### 2.1 Binding of melittin with EoCen

A solution of ME was added to dry fluorescence cuvette in 0.1 mol/L Hepes and 150 mmol/L NaCl at pH 7.4. With the addition of sequential aliquots of apoEoCen to the cuvette, fluorescence emission of ME (Figure 1-3) was shifted from 350 to 335 nm (Figure 1-5), indicating that hydrophilic microenvironments of Trp in ME were changed significantly. Burstein et al.[9] thought the fluorescence of Trp was differently located in different solves. And fluorescence intensity of ME was increased by 1.2-fold compared with that of ME indicating a shielded environment for Trp residue in ME. At the same time, fluorescence spectra of apoEoCen as well as holoEoCen were scanned as control solutions shown in Figure 1-1, 2, respectively. It can be seen that the apoEoCen *plus* ME spectrum (Figure 1-4) is different from that of ME alone and far from the spectrum of complex apoEoCen-ME, suggesting that complex has been formed in the absence of $Ca^{2+}$.

In addition, interaction between EoCen and ME was monitored in 0.1 mol/L Hepes and 150 mmol/L NaCl at pH 7.4 by native PAGE (Figure 2(a)). With the ratio of ME to EoCen increasing, there appeared a new protein band in lanes 2-6 with mobility slightly slower than that

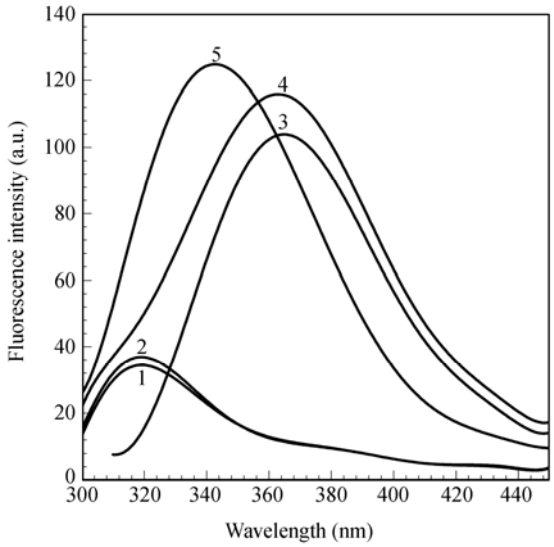


**Figure 1** Centrin-ME interaction measured by the Trp fluorescence of ME in 0.1 mol/L Hepes and 150 mmol/L NaCl, pH 7.4. Slits are 10 nm. Excitation is at 295 nm. Samples contain $8.6\times10^{-5}$ mol/L of the individual compounds or the equimolar mixtures. 1, apoEoCen; 2, holoEoCen; 3, ME; 4, apoEoCen *plus* ME; 5, apoEoCen-ME.

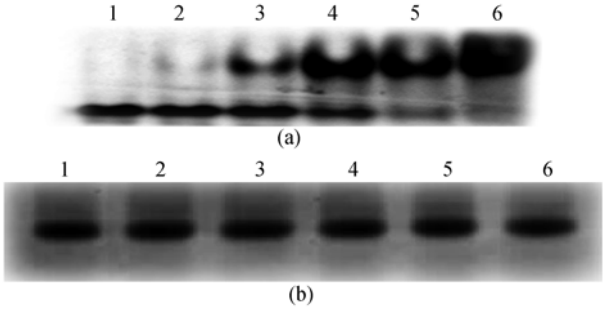


**Figure 2** (a) Native PAGE of ME binding with EoCen in the presence of $Ca^{2+}$. All lanes contained 1.05 nmol of EoCen and ME in increasing ratios as indicated: the ratio of ME to EoCen is (1) 0, (2) 0.20, (3) 0.40, (4) 0.84, (5) 0.98, (6) 3.0. Free ME does not migrate to the cathode in this native system. (b) Native PAGE of ME binding with N-EoCen and LC-EoCen in the presence of 1 mmol/L $Ca^{2+}$. All lanes contained 1.74 nmol of N-EoCen or LC-EoCen in increasing ratios as indicated: the ratio of ME to N-EoCen is (1) 0, (2) 1.10, (3) 3.40; the ratio of ME to LC-EoCen is (4) 0, (5) 1.12, (6) 3.0.

of EoCen indicating forming new complex, among which the positively charged ME alone cannot enter the gel. And this new band reaches maximal intensity at equimolar concentration of both proteins (lane 5 in Figure 2(a)), suggesting the formation of an ME-EoCen complex. No other complexes of different stoichiometries could be observed. ME bond with EoCen at the ratio of 1∶1 under the experimental conditions. Different from Human centrin 3 (HsCen3), both HsCen2 and EoCen formed 1∶1 centrin-peptide complex[10]. Similar conclusion has been reported on Chlamydomonas reinhardtii caltractin (CRC) that it is capable of binding simultaneously two amphiphilic peptides derived from yeast Kar1p protein[11]. In addition, interactions between ME and N-EoCen or LC-EoCen were also investigated by native PAGE. As shown in Figure 2(b), no new ME-EoCen band can be seen in despite of ratio of ME to EoCen increasing to 3.0, indicating inability of ME binding with N-EoCen or LC-EoCen.

CD was used to characterize the distribution of secondary structural elements in EoCen and determine whether any changes occurred upon binding ME with the protein. As shown in Figure 3, signals of CD of EoCen were increased significantly with the addition of ME in 10 mmol/L Hepes and 150 mmol/L NaCl at pH 7.4. As in general cases for linear polypeptides of this size, ME was highly disordered in 10 mmol/L Hepes and 150 mmol/L NaCl at pH 7.4 in the absence of EoCen (Figure 3-1). The far-UV CD spectrum of the EoCen showed two minima peaks at 207 and 222 nm (Figure 3-2), which is typical of a protein with a significant α-helical secondary structure[12]. With the addition of ME, there appeared a spectrum more negative than the sum of each taken separately, indicating that the interaction enhanced the α-helical content of the complex shown in Figure 3-3.

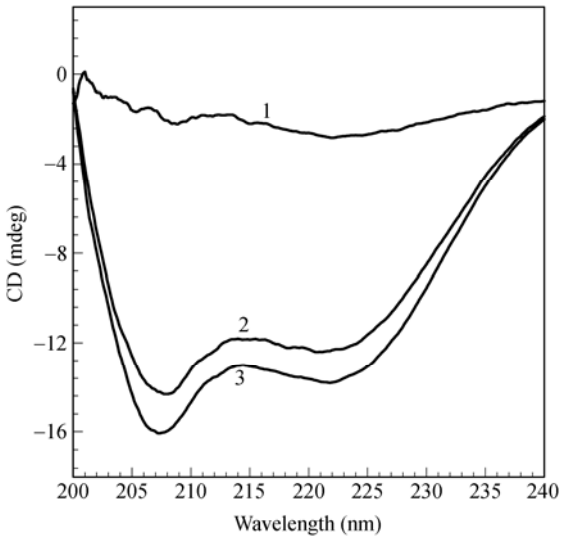


**Figure 3** Far-UV CD spectra of $2.5\times10^{-6}$ mol/L ME in 150 mmol/L NaCl and 10 mmol/L Hepes at pH 7.4 at 298 K in the presence of centrin. 1, ME: ($2.01\times10^{-4}$ mol/L); 2, apoEoCen: ($5.60\times10^{-5}$ mol/L); 3, ME-EoCen: (ME: $5.60\times10^{-5}$ mol/L, EoCen: $5.60\times10^{-5}$ mol/L).

## 2.2 Domain bound melttin

To correct the dilution effect, fluorescence intensity at 335 nm was converted to molar fluorescence intensity

($F_M$) via dividing fluorescence intensity by analytical concentration of ME. The plot of $F_M$ against $r$ ($r$ = EoCen/ME) is displayed in Figure 4-1. It can be seen that fluorescence intensity of the complex at 335 nm was increased significantly and then that was increased slightly after $r > 1:1$. Based on the titration curves, the conclusion that ME binds to EoCen at ratio of 1:1 can be drawn. The curvature allows the estimation of conditional binding constant $K_a$ to be ~$10^{6.81}$ L/mol according to published method[13] and corresponding free energy of 38.8 kJ/mol. In contrast with EoCen, addition of SC-EoCen into solution of ME brought out similar blue-shift and fluorescence increase (Figure 4-2). The titration curves for SC-EoCen showed a rapid increase in the fluorescence intensity indicating strong binding ($K_a = 10^{6.59}$ L/mol) with 1:1 stoichiometry and free energy of 37.6 kJ/mol, which represents 97% from that of EoCen.

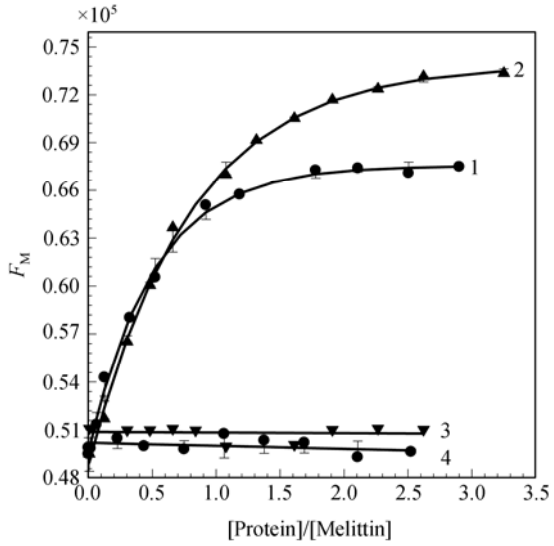


**Figure 4** Fluorescence titration curves for the addition of (1) EoCen ($8.6\times10^{-5}$ mol/L); (2) SC-EoCen ($7.1\times10^{-5}$ mol/L); (3) N-EoCen ($9.4\times10^{-5}$ mol/L); (4) LC-EoCen ($8.4\times10^{-5}$ mol/L) into ME ($6.9\times10^{-6}$ mol/L) in 0.1 mol/L Hepes and 150 mmol/L NaCl at pH 7.4 with excitation at 295 nm.

Different from EoCen and SC-EoCen, addition of N-EoCen and LC-EoCen to solution of ME induced mild blue shifts but no significant fluorescence enhancement and their titration curves are shown in Figure 4-3,4, respectively. This suggested strongly that interaction between EoCen and ME is mediated primarily by short C-terminal domain of EoCen. LC-EoCen exhibits a compact five-helix fold, in which the amphiphilic D-helix interacts strongly with the hydrophobic groove, created by the two EF-hand motifs. The inability of LC-EoCen to bind with ME probably resulted from the competition between D-helix and the peptide for the same binding pocket, the D-helix having a higher affinity.

Anisotropy measurements are ideally suited for measuring association of proteins with other macromolecules. With the addition of EoCen, anisotropy of ME was increased dramatically shown in Figure 5-1. The maximal anisotropy of ME increased until $r$ reached to 0.91, implying stoichiometry not greater than 1:1. Under the similar experimental conditions, anisotropy changes of ME have been measured with addition of SC-EoCen, LC-EoCen, and N-EoCen. Results suggested that anisotropy enhancement of SC-EoCen can be observed before $r < 1.0$ (Figure 5-2). However, no changes of anisotropy values can be observed while LC-EoCen and N-EoCen were added into solution of ME under the similar conditions (data not shown).

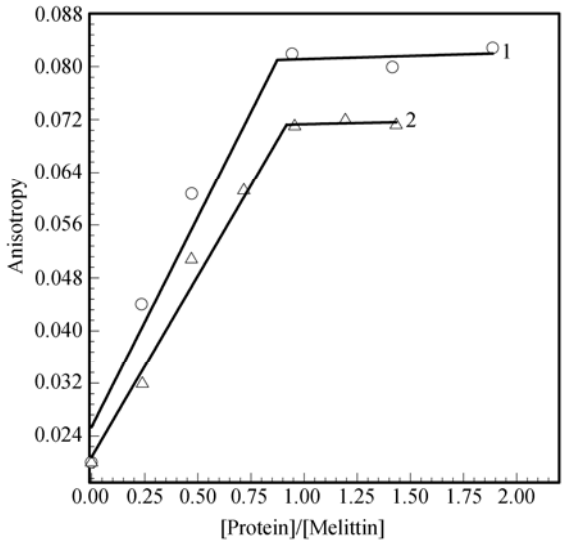


**Figure 5** Fluorescence anisotropy titration of peptide binding to EoCen (1) and SC-EoCen (2) in 0.1 mol/L Hepes and 150 mmol/L NaCl at pH 7.4 with excitation at 295 nm.

## 3 Conclusion

In the present paper, the interaction between EoCen and ME was studied by fluorescence spectra, CD spectra and native PAGE at neutral pH. Results suggested that EoCen interacted with ME exclusively by its C-terminal domain. Both EoCen and SC-EoCen may bind to ME with the 1∶1 stoichiometric ratio with affinity of ~$10^{-7}$ mol/L.

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