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Modulation effect of double strand DNA on the self-assembly of N-terminal domain of *Euplotes octocarinatus* centrin

Wenlong Zhang[a], Enxian Shi[a,b], Yaqin Zhao[a], Binsheng Yang[a,*]

[a] Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Taiyuan 030006, China
[b] Department of Pharmacy, Shanxi Medical University, Taiyuan 030006, China

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ABSTRACT

Centrin is a member of the EF-hand super family of calcium-binding proteins, which can behave as a part of damage detector initiated the initiation of nucleotide excision repair (NER). Its self-assembly plays a causative role in fiber contraction associated with the cell division cycle and ciliogenesis. To explore the possible role of DNA in the process of centrin self-assembly, the aggregation properties of N-terminal domain of *Euplotes octocarinatus* centrin (N-EoCen) in the presence of DNA with or without metal ions are investigated. It is verified that metal ions, such as Ca^{2+} and Tb^{3+}, can bind to N-EoCen with 2:1 stoichiometry by isothermal titration calorimetry (ITC). Importantly, this study reports that double strand DNA (dsDNA) is capable of binding N-EoCen, changing conformation of protein and modulating centrin aggregation, as demonstrated by extensive biophysical assays. Interestingly, the open conformation of protein induced by metal ions may be favour of the interaction of protein with dsDNA. Nevertheless, the randomly coiled single strand DNA (ssDNA) is completely inefficient to the aggregation regulation. Furthermore, results reveal that hydrophobic site could play important role in the process. This finding may link to the potent roles of centrin in the NER process.

1. Introduction

Centrin is acidic centrosomal or contractile fiber-associated protein, which belongs to the subfamily within the superfamily of Ca^{2+}-modulated proteins [1]. It has been found that centrin is ubiquitous in all eukaryote species, ranging from unicellular organisms to vertebrates cells, since it was first identified in unicellular green algae, such as *Tatraselmis striata* and *Chlamydomonas reinhardtii* [2].

Centrin is composed of two domains, N- and C-terminal domains, linked by a flexible central α-helix. The crystal structure of centrin reveals a dumbbell-shaped protein with two globular domains. In general, it contains four helix-loop-helix topology structures, the so-called EF-hand subdomains. The EF-hand structural motif was devised recognised in 1973 and is the most common calcium binding motif found in proteins [3]. Ca^{2+} ions are coordinated by ligands within the loop region (usually about 12 amino acids). Upon Ca^{2+} binding, EF-hand protein undergoes a large conformational change from “closed” to “open” and exposes more hydrophobic surface [4–6]. Besides, the presence of Ca^{2+} can lead to more spherical shaped structure from elongated shape of Ca^{2+}-free centrins. The amino-terminal subdomain of centrin, especially its first 20-residue fragment, is the most distinctive and variable region of centrin, which confers its functional diversity [7]. Ca^{2+}-dependent aggregation of the centrin is also mainly dependent on the amino-terminal subdomain. Research has shown that centrins can form multimers in the Ca^{2+} loaded state above a protein concentration of 10 μM [7].

As a major component of contraction fibers, centrin plays a fundamental role in various cellular processes [7–17]. Extensive researches have proved that centrin is tightly linked to the regulation of the microtubule organizing center (MTOC) duplication and separation during the cell cycle. And only about 10% of the centrins are concentrated on the MTOC [1]. Centrin distributed throughout the cell is strongly associated with the light transduction cascade in photoreceptor cells [2], the nuclear mRNA export in yeast and the ciliary voltage-gated Ca^{2+} channel in *Paramecium* in *Tetrahymena* [18,19]. Moreover, centrin may play a functional role in pre-mRNA splicing [14] and regulation of neuron-specific K^{+} channel [15]. Recently, related investigations have reported the participation of centrins in a recognition process in nucleotide excision repair (NER) [20–22]. Xeroderma pigmentosum group C (XPC) protein, the human homologue of Rad23B (HR23B) protein and centrin ternary complex behaves as a damage detector that initiated the global genome NER. However, it is still unclear about the molecular mechanisms implicated in the process. Does it involve in centrin aggregation? Hence, it is of particular significance to understand centrin

* Corresponding author.
E-mail address: yangbs@sxu.edu.cn (B. Yang).

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aggregation in the presence of DNA with or without metal ions, which may provide new insights into the multifunctionality of centrin.

Euplotes octocarinatus centrin (EoCen) is firstly reported by our laboratory [23] (gene register Y18899), which is obtained from *Euplotes Octocarinatu*. It contains no tryptophan and four tyrosine residues. N-terminal domain of EoCen (N-EoCen) is composed of first two EF-hands and involves in 101 amino acid residues. Indeed, our previous work has suggested that N-terminal domain play a critical role in the process of EoCen self-assembly [24–26]. It has been found that Ca^{2+} not only binds to N-EoCen with 2:1 stoichiometry, but also induces protein to produce aggregates [24,25]. The order for the metal-binding affinity of the two sites in N-EoCen is site I > site II [27]. Tb^{3+}, as lanthanide ion, induces larger conformation change and leads to stronger aggregation, even if it has similar coordination chemistry properties with Ca^{2+} [24,25,28–30]. The average conditional binding constants for two sites are $(7.52 \pm 0.02) \times 10^2\ M^{-1}$ for Ca^{2+} and $(2.13 \pm 0.10) \times 10^5\ M^{-1}$ for Tb^{3+}, respectively [31]. The affinity of site II to Ca^{2+} is $(3.52 \pm 0.08) \times 10^2\ M^{-1}$, the result is $(1.61 \pm 0.04) \times 10^5\ M^{-1}$ for Tb^{3+} [32].

In this paper, we mainly characterize the self-assembly properties of N-EoCen with or without metal ions in the presence of DNA, by using native polyacrylamide gel electrophoresis (native-PAGE), resonance light scattering (RLS), Thioflavin T (ThT) fluorescence and 2,6-*p*-toluidino naphthalene sulfonate (TNS) fluorescence measurements. Results are extensively discussed and the modulation effect is explored to understand the interaction mechanism.

2. Experimental

2.1. Reagents

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), TNS, ThT, and DNA were received from Sigma. Tryptone, yeast extract, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) Calcium chloride ($CaCl_2$), bromophenol blue and Coomassie brilliant blue R-250 were purchased from Sangon in Shanghai of China. Biochemical reagents in construction, expression and purification of proteins were obtained from Trans Gene. Tb_4O_7 with a purity of 99.9% was used. The stock solution of terbium (0.0143 M) was prepared by dissolving weighed Tb_4O_7 in concentrated hydrochloric acid, which was then standardized by complexometric titration with ethylene diamine tetraacetic acid (EDTA) using xylenol orange as the indicator in acetic acid-sodium acetate (HAc/NaAc) buffer at pH 5.5. All other chemicals were the highest purity available from local sources.

2.2. Preparation of DNA

Stock solution of double strand DNA (dsDNA) was prepared in 10 mM Hepes. It was measured from the absorption at 260 nm (ε_{260} = 6600 $M^{-1}\ cm^{-1}$, T = 25 °C). The DNA solution exhibited an ultraviolet (UV) absorbance ratio (A_{260}/A_{280}) of 1.92, demonstrating that DNA was sufficiently free from protein [33]. The concentration measurements were performed on a Varian-Cary Eclipse UV–visible spectrophotometer. The stock solutions were used immediately after it was ready. Single strand DNA (ssDNA) was obtained by heating the dsDNA solution for 10 min in boiling water, followed by cooling in ice.

2.3. Protein preparation

Using full-length gene as template, N-EoCen was required by polymerase chain reaction (PCR) technique. The PCR product was subcloned into a pGEX-6p-1 vector. The clone result was confirmed by DNA sequence analysis. The recombinant plasmids were transferred into *Escherichia coli* strain BL21 (DE3) and incubated in Luria Bertzani (LB) media containing 100 μg/mL ampicillin at 37 °C. When the optical density was up to 0.6–0.8 (at 600 nm), protein was induced using 0.8 mM IPTG for 3.5 h. GST fusion proteins were purified using glutathione sepharose 4FF in PBS. Isolate N-EoCen protein was obtained by PreScission Protease (PPase) cleavage, AKTA purifier FPLC system and passing over a HiLoad™ 16/60 Superdex™ 200 gel filtration column. Detailed procedures were described in [26]. Protein was further identified via 15% SDS-PAGE (indicated > 99% purity). The purified proteins were conserved in 10 mM Hepes (pH 7.4) at − 80 °C. Protein concentration was measured using molar extinction coefficient at 280 nm of 4350 $M^{-1}\ cm^{-1}$.

2.4. Isothermal titration calorimetry assay

Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal iTC200 device (MicroCal, Northampton, MA, USA) at 25 °C. The protein, metal ions, and DNA were equilibrated in the same buffer containing 10 mM Hepes (pH 7.4). The instrument consists of two identical cells, one for the sample and one for the reference solution. The reference cell of the microcalorimeter was filled with ultrapure water, and both cells were maintained at the same temperature. Protein was deposited in the sample cell at 0.06–0.08 mM and was titrated with metal ions at 0.8–2.0 mM by automatic injections of 1 μL at 25 °C. As for protein-DNA interaction, protein deposited in the sample cell at 0.06 mM was titrated with DNA at 0.8–2.5 mM by automatic injections of 1 μL at 25 °C. The first injection of 0.4 μL was ignored in the final data analysis. The integration of the peaks corresponding to each injection and the correction for the baseline were performed with the Origin-based software provided by the manufacturer. The data were fit to an interaction model to generate the stoichiometry (n), equilibrium binding constant (K_a) and enthalpy of complex formation (ΔH). Typically, control experiments consisting of injections of metal ion solutions into the buffer were performed to evaluate the heat of dilution. At least three replicates were performed for each experiment. The results were analyzed and calculated by the MicroCal Origin Software (MicroCal, Northampton, MA, USA).

2.5. Fluorescence spectroscopy

All fluorescence experiments were performed on a Varian-Cary Eclipse fluorescence spectrometer. The excitation and emission slits were both 10 nm. The excitation wavelength were set at 320 nm for TNS, 450 nm for ThT and 290 nm for Tb^{3+} in steady-state fluorescence experiments. In Tb^{3+} fluorescence experiments, a 360 nm emission filter was used. The excitation wavelength was set at 280 nm for measuring the affinity of DNA to N-EoCen in the fluorimetric titrations experiments. The reported constants are averages of three experimental values.

2.6. Native-PAGE analysis

Samples (DNA, N-EoCen, and metal ions) were incubated in Hepes (10 mM, pH 7.4) at 4 °C for 12 h before addition of loading buffer (1/5 volume). The loading buffer consists of 50% glycerol and 0.5% bromophenol blue. Then electrophoresis was carried out in 15% native-PAGE (acrylamide/bis-acrylamide = 29:1) containing tris-glycine buffer. Gels ran at the constant potential of 80 V. Protein was visualized in gels by stained with Coomassie brilliant blue R-250 (0.25% Coomassie brilliant blue R-250, 45% methylalcohol, 10% glacialacetic acid) using ZHGP-70I film illuminator.

2.7. Resonance light scattering

Solution turbidity changes were monitored by the change of fluorescence at a wave length 367 nm of samples in 1 cm optical path quartz cells in 10 mM Hepes at pH 7.4 with a fluorescence spectrometer (F-2500, Hitachi, Japan). The slit widths of excitation and emission were both 5 nm, using the same emission and excitation wavelengths