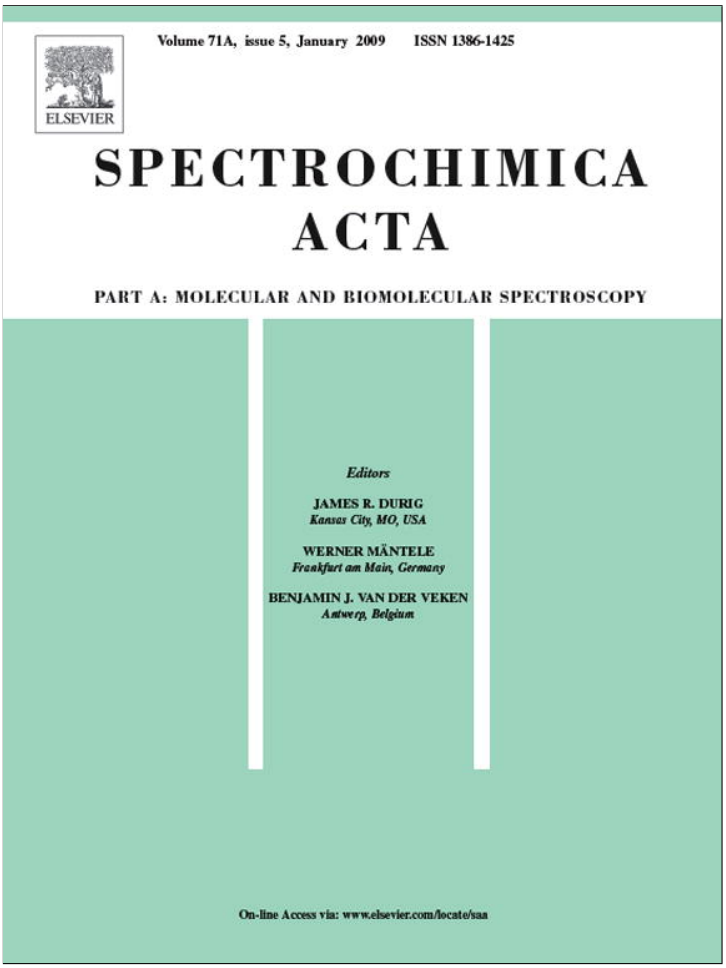

Spectrochimica Acta Part A 71 (2009) 1756–1761

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

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# The characterization for the binding of calcium and terbium to *Euplotes octocarinatus* centrin

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ARTICLE INFO

*Article history:*
Received 18 January 2008
Received in revised form 11 June 2008
Accepted 25 June 2008

*Keywords:*
$Ca^{2+}$
$Tb^{3+}$
Centrin
Spectra

ABSTRACT

Centrin is a member of the EF-hand superfamily that plays critical role in the centrosome duplication and separation. In the present paper, we characterized properties of metal ions binding to *Euplotes octocarinatus* centrin (EoCen) by fluorescence spectra and circular dichroism (CD) spectra. Changes of fluorescence spectra and α-helix contents of EoCen proved that $Tb^{3+}$ and $Ca^{2+}$ induced great conformational changes of EoCen resulting in exposing hydrophobic surfaces. At pH 7.4, $Ca^{2+}$ (and $Tb^{3+}$) bond with EoCen at the ratio of 4:1. Equilibrium experiment indicated that $Ca^{2+}$ and $Tb^{3+}$ exhibited different binding capabilities for C- and N-terminal domains of protein. C-terminal domain bond with $Ca^{2+}$ or $Tb^{3+}$ ~100-fold more strongly than N-terminal. Aromatic residue-sensitized $Tb^{3+}$ energy transfer suggested that site IV bond to $Tb^{3+}$ or $Ca^{2+}$ more strongly than site III. Based on fluorescence titration curves, we reckoned the conditional binding constants of EoCen site IV quantitatively to be $K_{IV} = (1.23 \pm 0.51) \times 10^8\ M^{-1}$ and $K_{IV} = (6.82 \pm 0.33) \times 10^5\ M^{-1}$ with $Tb^{3+}$ and $Ca^{2+}$, respectively. Metal ions bond to EoCen in the order of IV > III > II, I.



## 1. Introduction

Centrins are small molecular (~20 kDa) calcium binding proteins well-identified in cells from protozoa and yeast to plant and human [1,2]. So far, three human centrin isoforms (HsCen1 to HsCen3) have been identified with variable sequences and different tissues and cell distributions [3–5]. Structurally, one centrin molecule contains four helix-loop-helix motifs called EF-hands, one pair of which is capable of binding two calcium ions ($Ca^{2+}$) [6]. The binding of calcium ions may involve structural rearrangement of α-helices with the consequent exposure of hydrophobic cleft, often referred as the "$Ca^{2+}$" switch [7]. By virtue of central roles in translating calcium signals into metabolic or mechanical responses, as well as in uptake, transport, and homeostasis of $Ca^{2+}$, the EF-hand calcium-binding proteins have been the subject of investigation by high resolution structural techniques [8]. Centrins cloned from different organisms contain different metallic binding sites. *Chlamydomonas* caltractin (CRC) has two high-affinity and two low-affinity $Ca^{2+}$ binding sites [9]. However, HsCen2 contains only one $Ca^{2+}$ binding site [10], different from which HsCen3 contains one $Ca^{2+}/Mg^{2+}$ mixed binding site except two specific $Ca^{2+}$ binding sites [11]. Different metal ions binding properties may induce various biological functions of EF-hand proteins.

Among the most centrins, CRC, HsCen2, and HsCen3 were the subjects of more detailed biophysically studies. Centrins in different organisms share high overall sequence homology as well as structural similarity. And, the subtle differences of primary structure in centrins are enough to result in distinct biochemical properties.

Over past years, the number of three-dimensional structures of centrins and centrin-peptides has increased at a very rapid pace. Like calmodulin (CaM), centrin is comprised of two globular domains connected by a central linker and each domain contains one pair of EF-hands which have the potential to bind two $Ca^{2+}$ ions [11]. N-terminal domain of centrin reveals a compact core conformation including four almost antiparallel α-helices and a short β-sheet. And the α-helices within EF-hands were perpendicular, which was regarded as characteristic of the $Ca^{2+}$-bound (open) state of regular $Ca^{2+}$-binding proteins [12]. Structural characterization of centrin revealed significant differences in the conformational dynamics of the N- and C-domains, which appear to influence their independent functions [13]. N-terminal domains of centrins function as the sensor of calcium signals and C-terminal domains of centrins serve as a constitutive anchor to target proteins in biological system [14]. In order to understand transduction of $Ca^{2+}$ signals by EF-hand proteins, it is important to recognize the affinity for $Ca^{2+}$ and conformational response to the ions [15].

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doi:10.1016/j.saa.2008.06.029

*Euplotes octocarinatus* centrin (EoCen), cloned firstly by our groups (GenBank accession number: Y18899), belongs to the EF-hand calcium-binding protein containing 168 residues, which contains three Tyrosine (Tyr) residues in N-terminal (Tyr46, Tyr72, Tyr79) and one Tyr in C-terminal (Tyr168). It may also be comprised of two structurally independent globular domains connected by a flexible linker, each domain containing two helix- loop-helix calcium binding motifs.

To shed light on the functional significance of the high level of diversity in biological functions of EoCen, a comprehensive molecular characterization of the different polypeptides is required. To this end, we expressed and purified EoCen, M-EoCen (mutant EoCen at G151W) and C-M-EoCen (isolated C-terminal domain of mutant EoCen at G151W). In the present work, the molecular properties of EoCen, including $Ca^{2+}$/$Tb^{3+}$ binding abilities and $Ca^{2+}$/$Tb^{3+}$-induced conformational changes were studied by fluorescence spectra and circular dichroism (CD) spectra.

## 2. Experimental

### 2.1. Materials

*N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) was reagent grade and got from Sigma Ltd. $KH_2PO_4$, $Na_2HPO_4$, KCl, NaCl, $CaCl_2$ and ethylenediaminetertraacetic acid tetrasodium salt (EDTA) were all analytical reagents and purchased from Sangon in Shanghai.

Terbium oxide was 99.99% and purchased from Hunan in China. $Tb^{3+}$ solution was prepared as described previously [16].

Tryptone, yeast extract, ampicillin and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Amresco Ltd.

Other biochemical reagents in construction, expression and purification of proteins were purchased from TaKaRa.

### 2.2. Preparation of proteins

Three constructs of EoCen were used in this study, namely, EoCen (the wild type EoCen 1M-168Y), M-EoCen (mutant EoCen at G151W 1M-168Y) and C-M-EoCen (isolated C-terminal domain of mutant EoCen at G151W 90T-168Y). Mutant of G at 151 to W was carried out by site directed mutagenesis [17].

Clones for expression of M-EoCen and C-M-EoCen have been obtained by PCR amplification using the custom designed primers. The newly synthesized DNA was cloned to vector pGEM-T Easy and subsequently transformed into *Escherichia coli* (DH5α). After verification by DNA sequence analysis and sub-cloning into expression vector pGEX-6p-1, the fused GST-proteins were over-expressed at 37 °C and induced for 3 h by IPTG using the BL21 cell line in $LB_{AMP}$ [18]. Fusion proteins (M-EoCen and C-M-EoCen) were first purified as a GST fusion protein using glutathione sepharose 4FF in PBS (in mM): $KH_2PO_4$ 1.8, $Na_2HPO_4$ 10, KCl 2.7 and NaCl 140 at pH 7.4. The GST fusion proteins were then cleaved by PreScission Protease (PPase) and the cleaved proteins were further purified by HPLC. The purity of the samples was assessed by SDS-PAGE and reverse-phase HPLC. The concentration of proteins were measured by its absorption at 280 nm with an extinction coefficient of $\varepsilon_{280} = 5600\,M^{-1}\,cm^{-1}$ (EoCen), $\varepsilon_{280} = 11200\,M^{-1}\,cm^{-1}$ (M-EoCen) and $\varepsilon_{280} = 7000\,M^{-1}\,cm^{-1}$ (C-M-EoCen). Recombinant EoCen was expressed and purified as described previously [17].

### 2.3. Metal removal

To remove contaminating bound cations, the protein samples were first pretreated with EDTA and then passed through a 40 cm × 1 cm Sephadex G-50/G-75 column equilibrated in buffer PBS, at pH 7.4.

### 2.4. Fluorescence spectra

Fluorescence spectra were recorded on a HITACHI F-2500 fluorescence spectrophotometer. Fluorescence intensities were measured with HITACHI 850 fluorescence spectrophotometers. $Tb^{3+}$ emission fluorescence spectra were acquired from 475–635 nm with excitation at 280 nm (EoCen) or 295 nm (M-EoCen and C-M-EoCen). The slit widths for excitation and emission were 10 nm. A filter with a long pass of 290 nm (or 310 nm) was used to avoid secondary Raleigh scattering. Samples were prepared by gradually adding $Tb^{3+}$ solutions to the protein solutions. An equilibrium time of 5 min was used between each titration.

### 2.5. Circular dichroism spectroscopy

Samples were dissolved in 10 mM Hepes and 150 mM NaCl (pH 7.4) at 20 °C. CD spectra were recorded on a Jasco J-810 spectrometer equipped with a Peltier temperature control unit. All spectra represent the average of three scans over the range of 200–240 nm with a step size of 0.1 nm and a band width of 1 nm. The appreciative α-helix content of protein ($f_h$) was evaluated using the equation provide by Maulet and Cox [19]

$$f_h = -\frac{|\theta|_{222} + 2340}{30300} \quad (1)$$

## 3. Results and discussion

### 3.1. Fluorescence spectra

Lanthanide ($Ln^{3+}$) ions have similar ionic radii ($Tb^{3+}$ 0.923 Å and $Ca^{2+}$ 1.00 Å) and similar coordination properties to $Ca^{2+}$ [20]. Hence, $Tb^{3+}$ was usually used to sense properties of $Ca^{2+}$-binding proteins through energy transfer [21]. In the paper, fluorescence spectra were used to monitor $Tb^{3+}$ binding to EoCen in 0.1 M Hepes and 150 mM NaCl at pH 7.4. With the addition of $Tb^{3+}$, four characteristic peaks of $Tb^{3+}$ at 490 nm, 545 nm, 580 nm and 620 nm

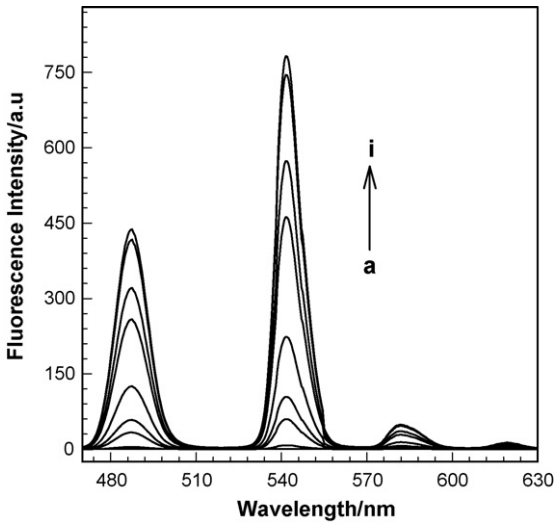


**Fig. 1.** Fluorescence spectra for the addition of $Tb^{3+}$ to the solution of EoCen ($4.3 \times 10^{-5}$ M) in 0.1 M Hepes and 150 mM NaCl at pH 7.4 with excitation at 280 nm. Slit widths 10 nm. The volume of $Tb^{3+}$ ($7.1 \times 10^{-4}$ M) is (b) 0 μL; (c) 10 μL; (d) 20 μL; (e) 30 μL; (f) 40 μL; (g) 60 μL; (h) 80 μL; (i) 100 μL and curve (a) represents the fluorescence spectra of $Tb^{3+}$ ($5.1 \times 10^{-3}$ M).

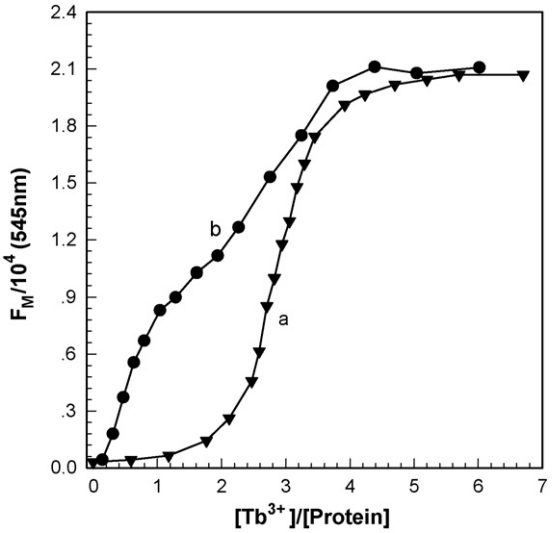


**Fig. 2.** Titration curves for the addition of $Tb^{3+}$ into (a) EoCen ($4.3 \times 10^{-5}$ M) and (b) M-EoCen ($1.6 \times 10^{-5}$ M) in 0.1 M Hepes and 150 mM NaCl at pH 7.4 with excitation at 280 nm (a) or 295 nm (b).

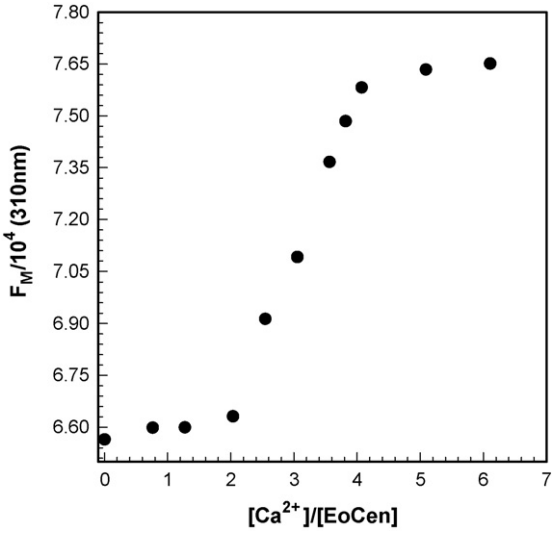


**Fig. 3.** Fluorescence titration curves of adding $Ca^{2+}$ to the solution of EoCen ($6.2 \times 10^{-5}$ M) in 0.1 M Hepes and 150 mM NaCl at pH 7.4 with excitation at 280 nm. Slit widths 10 nm.

appeared by virtue of aromatic residue-sensitized $Tb^{3+}$ energy transfer (Fig. 1). To correct dilution, the fluorescence intensity at 545 nm was converted to molar fluorescence intensity ($F_M$) by dividing the fluorescence intensity via the analytical concentration of EoCen. As a function of the molar ratio of $Tb^{3+}$ to EoCen ($r = [Tb^{3+}]/[Protein]$), changes in $Tb^{3+}$ fluorescence at 545 nm were plotted in Fig. 2 curve a. The titration curves revealed that two breaks appeared at near $r = 2$ and $r = 4$, confirming the 4:1 stoichiometric ratio of the $Tb_4$-EoCen complex and two types of binding sites of EoCen. One type of binding site was ascertained as high affinity site and the other low affinity site.

When suitable donor chromospheres, such as the aromatic ring of Tryptophan (Trp) residue or Tyr, is in proximity, $Tb^{3+}$ accepts the energy from the excited donor and fluorescence enhancement at 545 nm can be observed. There is no Tyr or Trp in the third or fourth EF-loops of EoCen. Therefore, weaker $Tb^{3+}$ fluorescence sensitization ($r \leq 2$) can be attributed to the binding of $Tb^{3+}$ to C-terminal binding sites of EoCen, whereas stronger $Tb^{3+}$ fluorescence sensitization ($2 \leq r \leq 4$) can be attributed to the binding of $Tb^{3+}$ to N-terminal binding sites of EoCen. That is to say that $Tb^{3+}$ bond to C-terminal domain of EoCen more preferentially than N-terminal of protein.

While Trp and Tyr at same time exist in protein the energy transfer will mainly happen between Trp and metal ions. Residue at position 151 did not bind with metal ions directly in calcium binding site IV. In addition, Gly and Trp contain neither positive nor negative charges. In the study, Gly residue of EoCen at 151 was mutated into Trp residue by site-directed mutagenesis, which was named as M-EoCen. CD measurement proved that no significant conformational changes arose from mutation (data not shown). So M-EoCen may be roughly used to distinguish metal-binding affinity between site III and IV by measuring the $Tb^{3+}$ fluorescence sensitization from 151Trp to $Tb^{3+}$. Fig. 2 curve b is the titration curve of M-EoCen produced by the addition of $Tb^{3+}$. As shown in Fig. 2b, breaks occurred near $r = 1$, 2, 4. Comparing Fig. 2 curve a, it can be concluded that affinity of $Tb^{3+}$ with site IV was stronger than with site III. Thus the affinity of EoCen with $Tb^{3+}$ accords with the order IV>III>II, I. The interaction of EoCen with $Ca^{2+}$ was monitored by fluorescence intensity of EoCen at 310 nm in 0.1 M Hepes and 150 mM NaCl at pH 7.4. The binding of $Ca^{2+}$ to EoCen led to fluorescence enhancement of protein to 122%. By similar data processing procedures, $Ca^{2+}$ titrating EoCen proved two low-affinity and two high-affinity binding sites in protein shown in Fig. 3, which was consistent with the conclusion drawn from $Tb^{3+}$ titrations (Fig. 1).

### 3.2. *Affinity of $Tb^{3+}$/$Ca^{2+}$ with EoCen*

Smaller ionic radii and stronger ionic potentials of $Lu^{3+}$ made it much easier binding to oxygen (O) or nitrogen (N) atom in the protein than $Tb^{3+}$. With the addition of $Tb^{3+}$ into the mixed solution of $Lu^{3+}$ and EoCen ($Lu^{3+}$:EoCen = 1.9:1.0), fluorescence intensity at 545 nm increased substantially by virtue of binding $Tb^{3+}$ to N-terminal domain of EoCen. As shown in Fig. 4, one break near $r = 2$ ($r = [Tb^{3+}]/[Lu_2\text{-EoCen}]$) can be observed, indicating that 2:1 stoichiometric ratio of $Tb^{3+}$ to $Lu_2$-EoCen were formed. On the basis of fluorescence titration curves, conditional binding constants of $Tb^{3+}$ with sites I and II in intact EoCen were measured to be $K_{I,II} = (2.01 \pm 0.10) \times 10^6\ M^{-1}$. $Ca^{2+}$ can make the fluorescence of $Lu_2$-EoCen-$Tb_2$ at 545 nm be quenched. Assuming

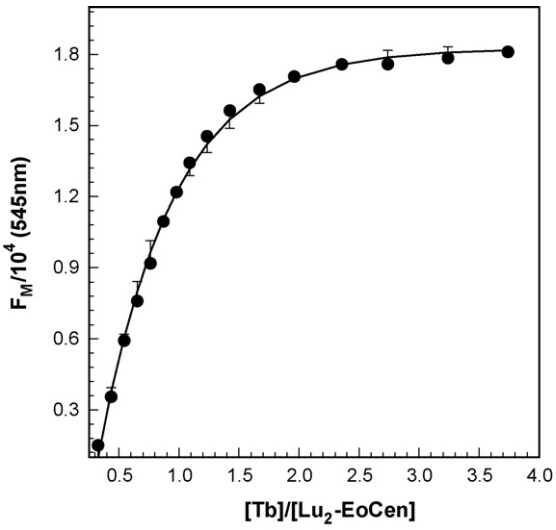


**Fig. 4.** Changes of fluorescence intensity with adding $Tb^{3+}$ ($6.4 \times 10^{-4}$ M) into solution of $Lu_2$-EoCen ($4.3 \times 10^{-5}$ M) in 0.1 M Hepes and 150 mM NaCl at pH 7.4 with excitation at 280 nm and slit widths 10 nm.

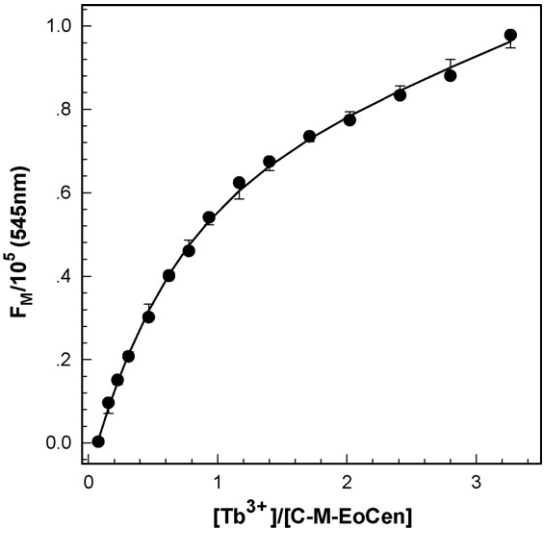


**Fig. 5.** $Tb^{3+}$ binding curves of solution C-M-EoCen ($1.69 \times 10^{-5}$ M) obtained from fluorescence spectroscopy in 150 mM NaCl and 0.1 M Hepes at pH 7.4 with excitation at 295 nm. Slit widths 10 nm.

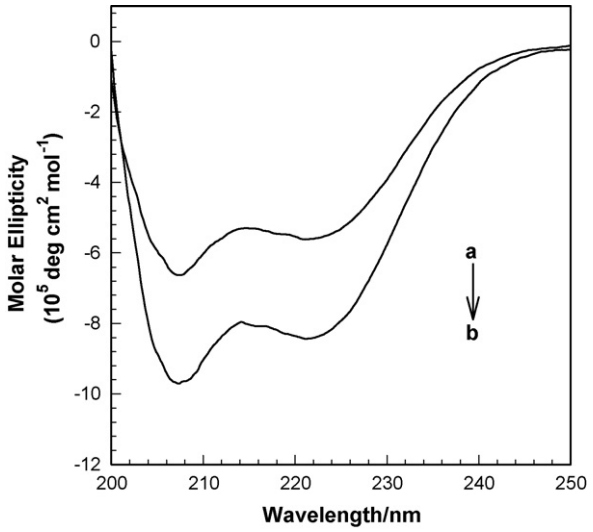


**Fig. 6.** Far-UV light CD spectra of 2.5 μM EoCen in 10 mM Hepes and 150 mM NaCl at pH 7.4 at 298 K in the presence of $Ca^{2+}$. (a) apoEoCen, (b) $Ca_2{}^{2+}$-EoCen.

that the fluorescence quenching comes from the competition of binding sites between $Ca^{2+}$ and $Tb^{3+}$, the binding constants of $Ca^{2+}$ with sites I and II in intact EoCen were measured to be $K_{I,II} = (1.12 \pm 0.04) \times 10^3\ M^{-1}$ by published method [22]. The binding constants either $Tb^{3+}$ or $Ca^{2+}$ in intact EoCen are greater than that in semi-molecule centrin (N-EoCen), meaning that there exists cooperativity between N-terminal and C-terminal domains in EoCen.

In order to avoid disturbance from N-terminal domain and introducing luminescent residue, C-M-EoCen, isolated C-terminal mutant EoCen covering one Trp at 151 in site IV and no any other luminescent residues was used to assess the affinity of site IV with $Tb^{3+}$ in 0.1 M Hepes and 150 mM NaCl at pH 7.4. Fig. 5 is the titration curve of C-M-EoCen, produced by plotting $F_M$ at 545 nm vs. $r$ ($r = [Tb^{3+}]/[C\text{-}M\text{-}EoCen]$). As shown in Fig. 5, larger sensation at $Tb^{3+}$/ C-M-EoCen $\leq$1:1 can be attributed to the binding of $Tb^{3+}$ to site IV of C-M-EoCen. There is no Trp or Tyr in site III of C-M-EoCen. So the fluorescence intensity of $Tb^{3+}$ at 545 nm is very small in the binding of $Tb^{3+}$ to site III of C-M-EoCen. The conditional binding constant of $Tb^{3+}$ with site IV in C-M-EoCen in 0.1 M Hepes and 150 mM NaCl at pH 7.4 can be calculated by fitting the metal titration curves with the following equations modified from binding process [23].

$$[\text{protein}]_t \times f^2 - ([\text{protein}]_t + [Tb^{3+}]_t + K_d) \times f + [Tb^{3+}]_t = 0 \quad (2)$$

$$f = \frac{F_M - F_M(0)}{F_M(\text{max}) - F_M(0)} \quad (3)$$

where $[\text{protein}]_t$ and $[Tb^{3+}]_t$ are the total concentration of C-M-EoCen and $Tb^{3+}$, respectively. $F_M(0)$ and $F_M$ are the molar fluorescence intensity in the absence and presence of $Tb^{3+}$, respectively. $F_M(\text{max})$ is molar fluorescence intensity of EoCen which was saturated by $Tb^{3+}$. And affinity of $Tb^{3+}$ to site IV in EoCen can be inferred to be $K_{IV} = (1.23 \pm 0.51) \times 10^8\ M^{-1}$.

Metal ion $Tb^{3+}$ can be used as competition experiments to determine the relative binding constant of $Ca^{2+}$ with EoCen [22]. Through similar method, the conditional binding constant of $K_{IV}$ ($Ca^{2+}$-protein) in intact EoCen was calculated to be $(6.82 \pm 0.33) \times 10^5\ M^{-1}$.

### 3.3. $Tb^{3+}/Ca^{2+}$-induced conformational changes

The CD spectroscopy is an efficient method for a general structural characterization of proteins in solution. As shown in Fig. 6, the far-UV CD spectra of EoCen in the absence of $Ca^{2+}$ shows two minima at 208 and 222 nm, which was typical characterization for a protein rich in α-helix [24].

Comparing holoEoCen with the blank solution of apoEoCen, addition of $Ca^{2+}$ induces a 40% increase in the overall CD intensity, probably resulting from $Ca^{2+}$-induced increase in the α-helix content and decrease in the conformational flexibility of the protein. Similar phenomena can also be obtained while $Tb^{3+}$ was added into solution of apoEoCen shown in Fig. 7. The CD observations indicated that $Ca^{2+}$ or $Tb^{3+}$ binding could induce secondary structure changes of EoCen.

As a kind of useful probe of conformational changes, TNS was used for investigating hydrophobic patches on the accessible surface of protein [25]. ApoEoCen enhanced the fluorescence intensity

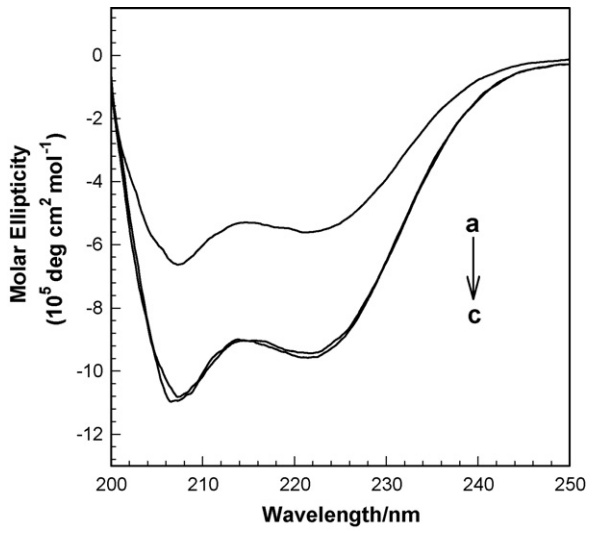


**Fig. 7.** Far-UV light CD spectra of 2.5 μM EoCen in the presence of $Tb^{3+}$ in 10 mM Hepes and 150 mM NaCl at pH 7.4 at 298 K. The ratio of $Tb^{3+}$ to EoCen was (a) 0, (b) 2.0, (c) 4.0.

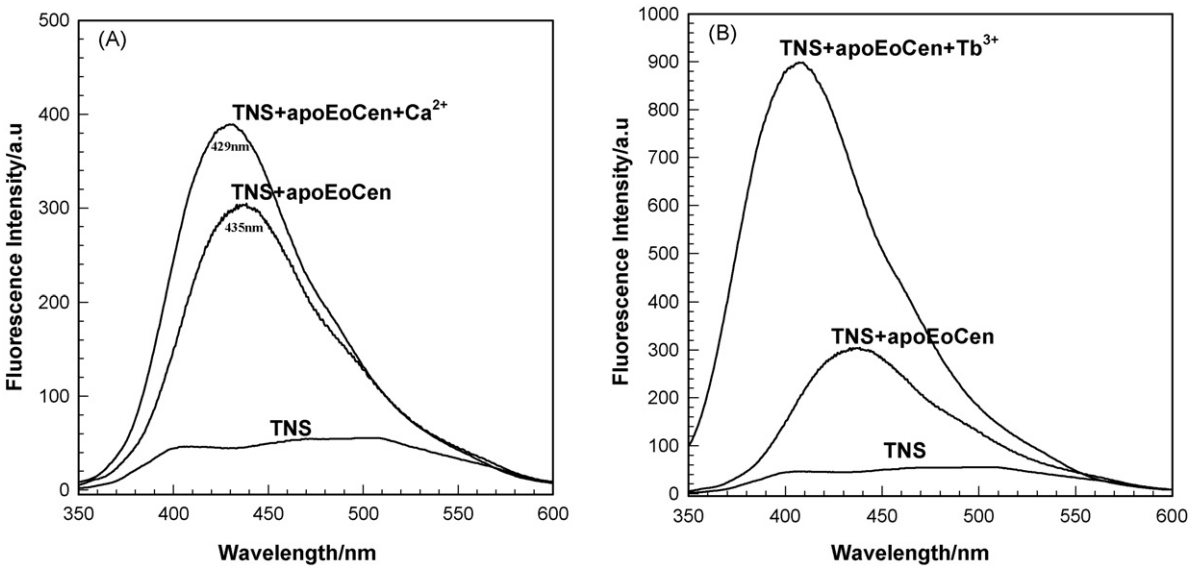


**Fig. 8.** $Ca^{2+}$ (A) and $Tb^{3+}$-dependent (B) exposure of hydrophobic side chains. Hydrophobic exposure was monitored at room temperature in 0.1 M Hepes and 150 mM NaCl (pH 7.4) with the hydrophobic TNS.

of the hydrophobic probe TNS 4-fold with a maximum at 435 nm (Fig. 8). Further fluorescence enhancement to 6-fold and blue-shift to 429 nm can be observed while $Ca^{2+}$ was added into mixed solution of TNS and apoEoCen. $Tb^{3+}$ has been widely used to sense $Ca^{2+}$-binding proteins [26-28]. In contrast, binding of $Tb^{3+}$ led to 13-fold fluorescence increase compared to that of the apo form and blue-shift of 27 nm. The instantaneous fluorescence changes upon binding of $Ca^{2+}/Tb^{3+}$ suggest that holo protein did not adopt a closed conformation and $Ca^{2+}/Tb^{3+}$-loaded centrin exposes more hydrophobic surface.

Proteins of EF-hand super-family are characteristic of their diversity in cation binding properties including affinity, kinetics, specificity and cooperativity. Centrins from different organisms have different metal ion binding properties including one or more non-functional binding sites. HsCen2 has unique high-affinity binding site localized in binding loop IV [10], while CRC show four high affinity binding sites, the strongest being localized in the N-terminal domain [9]. There exists three Ca2+ binding sites and one mixed $Ca^{2+}/Mg^{2+}$ site on HsCen3 [11]. As far as EoCen, it binds to two calcium ions with high ($\sim 10^5\ M^{-1}$) affinities and two calcium ions low affinities ($\sim 10^3\ M^{-1}$). On the basis of numerous experiments, one rule for evaluating binding affinity of metal ions with loops in EF-hand was reported [29]. By analyzing primary sequence (gene register Y18899), we found that site IV of EoCen is meet all of the criteria for classification as standard EF-hands, i.e. oxygen-bearing carboxyl side chains are present in the critical positions for $Ca^{2+}$ binding and segments with a propensity to form α-helices surrounded the $Ca^{2+}$-bindng loop. Hence, that site IV was recognized as the high-affinity site of EoCen is reasonable.

Calcium-induced exposure of hydrophobic surfaces has been shown to be a key step in signal transduction by EF-hand $Ca^{2+}$ sensor proteins [30]. In this sate, HsCen3 can bind its target peptide. For EoCen, conformational transition from a closed state to an open state clearly occurs providing for its functions when loops of EF-hand were occupied by $Ca^{2+}$ (strong TNS enhancement). Paper indicated that other EF-hands proteins including calexcitin [31], Nerieis SCP [32,33] and HsCen3 [11] display an open state in the calcium-saturated forms. In the form of calcium-binding state, EF-hand proteins may avoid degradation by the protease. Hence, the calcium-binding sites under physiological conditions are always occupied by divalent cations and the proteins are protected form the closed state, even at rest. In addition, metal ions binding to centrin may be the trigger for the interaction of protein with target peptide [10] and it also may provide some insights into the molecular mechanism of centrin functions in the cell.

## Acknowledgements

The project supported by the National Natural Science Foundation of China (no. 20771068) and the Shanxi Provincial Natural Science Foundation for Youth (no. 2007011024). We are grateful to Dr. Zhepeng Wang for the suggestions and Dr. Lianzhi Li for help in CD spectropolarimeter measurements.

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