

## Spectral study on the interaction of *ciliate Euplotes octocarinatus* centrin and metal ions

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### Abstract

Centrin is a low molecular mass (20 kDa) protein that belongs to the EF-hand superfamily of calcium-binding proteins. Centrin and calmodulin apparently function in distinct calcium signaling pathways despite substantial sequence similarity. To understand how centrin function, the glycine in position 115, the sixth amino acid of the loop of the protein's third EF-hand, was converted into tryptophan (Trp). Local and overall changes were monitored for interactions between cations and *Euplotes* centrin and the mutant using fluorescence and UV spectroscopies. The results show that the binding of four Ca<sup>2+</sup> ions to centrin with two higher affinity (sites III and IV) and two lower affinity (sites I and II), and the relative affinity of site IV is higher than that of site III. The secondary structure change of centrin and the mutant resulting from metal ions binding was measured by CD spectra. Centrin undergo a conformation change induced by Ca<sup>2+</sup> binding from "closed" to "open", through 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) as a probe, the different contributions to conformation change from the different sites were investigated, providing some insights into the molecular mechanism of centrin functions in the cell.

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**Keywords:** Centrin; Tb<sup>3+</sup>; Conformation change

### 1. Introduction

Centrins, also known as caltractin, are a closely related subfamily within the larger superfamily of Ca<sup>2+</sup>-binding proteins that includes calmodulin, troponin C and parvalbumin. These proteins contain four helix-loop-helix domains, the so-called EF-hands, which may each bind one Ca<sup>2+</sup> [1], as shown in Fig. 1a and b [2]. Since the discovery of caltractin in the green

alga *Tetraselmis striata* [3], homologs of the protein have been identified in a wide variety of organisms ranging from protozoa and yeast to plants and humans [4,5]. In higher eukaryotes, centrin has been shown to localize to the centrioles in the centrosome, which is the microtubule organizing center in these cells. In lower organisms, centrin is found in analogous structures, such as the basal body in algae and the spindle pole body in yeast. Genetic studies have shown that centrin is required for proper cell division. In *Chlamydomonas reinhardtii*, centrin is thought to play an important role in flagellar excision [6,7]. *C. reinhardtii* caltractin and its yeast homolog, cdc31p, have been shown to play an essential role during the cell cycle dependent duplication and separation of the microtubule organizing center in their respective cells [8–10].

*Euplotes* centrin is a protein of 168 residues, which shares about 60, 62 and 66% sequence identity with human centrin 1, human centrin 2 and human centrin 3, respectively, and shares approximately 50% sequence identity with the well studied

**Abbreviations:** Cen, *ciliate Euplotes octocarinatus* centrin; G115W, the mutant protein at 115 position; PBS, potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>, 1.8 mM), sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>, 10 mM) and potassium chloride (KCl, 2.7 mM), sodium chloride (NaCl, 140 mM); IPTG, isopropyl-*D*-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid

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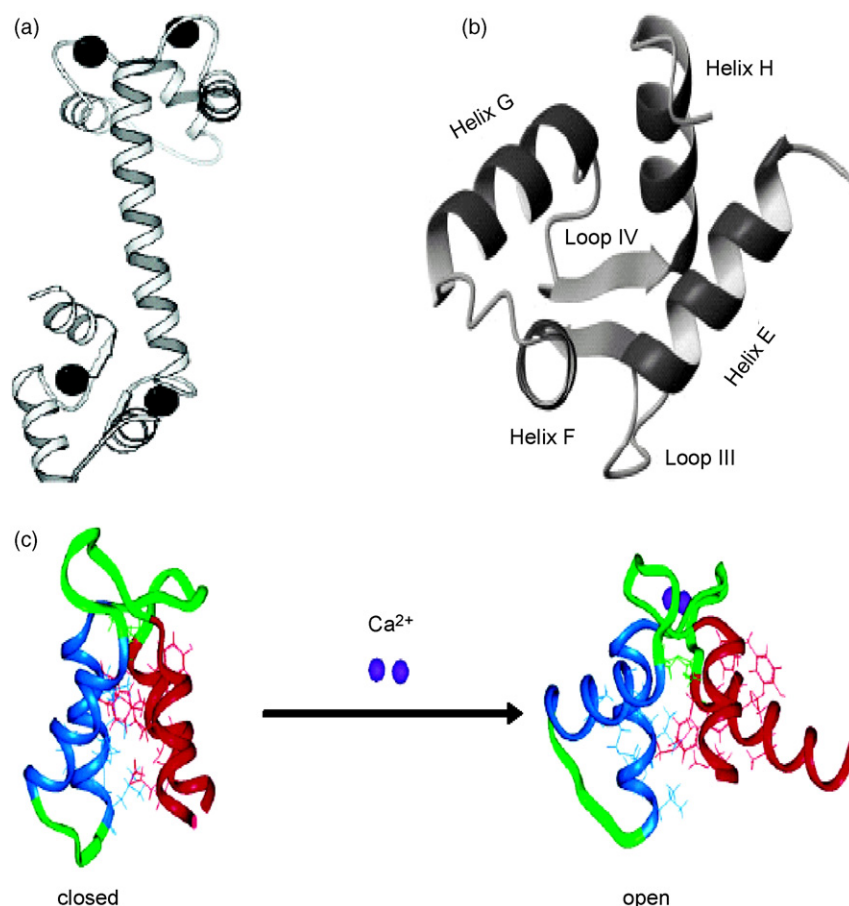


Fig. 1. (a) Three-dimensional structure of centrin represented by calmodulin [2]. Ca<sup>2+</sup> ions are shown as dark spheres (a three-dimensional structure of intact centrin is not yet available). (b) The diagram shows ribbon representations of the C-terminal of centrin. (c) Conformational switch of a representative EF-hand domain upon Ca<sup>2+</sup> binding [14]. This movement exposes the hydrophobic core of the domain, which can then interact with a protein partner.

EF-hand protein calmodulin. Like CaM, centrin consists of two independent domains tethered by a flexible linker, each domain comprising a pair of EF-hand motifs of helix-loop-helix that can potentially bind two calcium ions [11]. The 12-residue loop is responsible for the binding and coordination of the metal ions in the EF-hand loops. The Ca<sup>2+</sup> ion is coordinated to seven oxygen ligands from the side chains of residues 1, 3, 5, and 12, the main chain carbonyl of position 7, and a bridged water at position 9. These oxygen atoms are spatially coordinated to Ca<sup>2+</sup> in a pentagonal bipyramidal (or distorted octahedral) configuration [12,13]. As can be seen in Fig. 1c [14], upon Ca<sup>2+</sup> binding, the trigger proteins (Ca<sup>2+</sup>-modulated proteins or sensor proteins such as CaM and troponin C) undergo a large conformational change and in turn regulate a vast number of target proteins [15–17]. The readout of intracellular calcium signals must be very finely tuned to effect a rapid response to the transient and rather subtle (100-fold) variations in calcium ion (Ca<sup>2+</sup>) concentrations that constitute the calcium signals [18]. Understanding what structural factors and amino acid properties control Ca<sup>2+</sup>-induced conformational changes is a critical step toward understanding how sequence dictates EF-hand protein structure and function [19].

*ciliate Euplotes octocarinatus* centrin (Cen) is the first reported by our laboratory [20] (gene register Y18899), which is

cloned from *E. octocarinatus*, and the detailed biological function is unclear. In this report, through introducing an aromatic tryptophan residue, we have investigated the Ca<sup>2+</sup> binding characteristics and the affect of Ca<sup>2+</sup> on the conformation of this protein using Tb<sup>3+</sup> as a probe, owing to the same binding behaviors. Our results reveal the relation between metal ions binding features of Cen and amino acid sequence, and shed light on the mechanism of centrin functions in the cell.

## 2. Materials and methods

### 2.1. Reagents

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, salts, and other chemicals utilized in protein purification were reagent grade. TNS was purchased from Sigma.

### 2.2. Protein expression and purification

Cen was overexpressed off a PGEX-6p-1 plasmid construct in *Escherichia coli* BL21 (DE3) induced with isopropyl-D-thiogalactopyranoside (IPTG) to yield milligram quantities of the desired protein as reported previously [20]. Briefly, transformed *E. coli* cells were grown in LB media contain-

ing 100  $\mu\text{g}/\text{mL}$  ampicillin and incubated at 37 °C while monitoring its growth via OD measurements at 600 nm. Once OD600 reach at 0.6, a final concentration of 0.5 mM IPTG was added to the culture, and 3 h later, the cells were harvested and frozen. Frozen cells were thawed in PBS buffer and sonicated with a macro probe at mediate power on ice. This solution was centrifuged at 15,000  $\times g$  for 25 min at 4 °C. The supernatant was applied to a Glutathione-SepharoseTM 4B column equilibrated with proteins were washed out and eluted with PBS buffer. Fractions containing Cen were identified via SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

### 2.3. Construction and cloning of the mutant G115W

The sequence and the cloning strategy of the full-length gene of Cen have been described earlier [20]. Using the full-length gene as template, the G115W mutant clone was obtained by PCR, Primers are 5-ctgagaatttaagagaggtt-3 and 5-gcttatttcctgaattatc-3. The resultant PCR product was subcloned into a PGEX-6P-1 expression vector. The sequence of the clone was confirmed by commercial company. The mutant clone was transformed into BL21(DE3) cells for expression and purification in the similar way to the wide type Cen.

### 2.4. Metal ion removal and protein concentration

The protein samples were ultrafiltrated extensively against 10 mM Hepes, pH 7.4 containing 1 mM ethylenediamine- $N,N,N',N'$ -tetraacetic acid (EDTA) to remove  $\text{Ca}^{2+}$ . The protein concentration was measured spectrophotometrically using molar extinction coefficients at 280 nm of 5600  $\text{M}^{-1} \text{cm}^{-1}$  for Cen and 11490  $\text{M}^{-1} \text{cm}^{-1}$  for the mutant G115W. The extinction coefficient of centrin was estimated from the tyrosine (Tyr) and Trp content as described by Pace et al. [21].

### 2.5. Aromatic residue-sensitized $\text{Tb}^{3+}$ energy transfer

Aromatic residue-sensitized  $\text{Tb}^{3+}$  energy transfer was carried out with Cen concentration of 5.48  $\mu\text{M}$  or the mutant G115W 9.75  $\mu\text{M}$  in 10 mM Hepes pH 7.4, 25 °C.  $\text{Tb}^{3+}$  emission fluorescence spectra were acquired from 500 to 600 nm with excitation at 280 nm. Both slit widths of excitation and emission were 10 nm. All fluorescence data was collected on Hitachi F-2500 or Hitachi 850.

### 2.6. Circular dichroism spectroscopy

CD measurements were conducted on a Jasco J-810 (Jasco Inc., Easton, MD) spectropolarimeter at 25 °C with constant stirring. Samples contained 10.2  $\mu\text{M}$  Cen or 10.23  $\mu\text{M}$  G115W in 10 mM Hepes at pH 7.4. For the calcium titration, small aliquots of a 2 mM  $\text{CaCl}_2$  solution were added to achieve calcium-to-protein molar ratios between 1:1 and 40:1. For the  $\text{Tb}^{3+}$  titration, small aliquots of 2.275 mM  $\text{TbCl}_3$  solution were added to protein in the range  $[\text{Tb}^{3+}]/[\text{protein}]$  ratio of 0–2. All spectra were

the average of three scans over the range 200–250 nm with a step size of 0.2 nm and a bandwidth of 1 nm.

### 2.7. Interaction of centrin and the mutant G115W with the hydrophobic probe TNS

Changes in the fluorescence properties of TNS were monitored by fluorometry. After incubation of 19.38  $\mu\text{M}$  Cen in 10 mM Hepes containing 21.12  $\mu\text{M}$  TNS for 5 min, small aliquots of a 227.5  $\mu\text{M}$   $\text{Tb}^{3+}$  standard solution were added. For the mutant G115W, the concentrations of protein and TNS are 91.98 and 19.12  $\mu\text{M}$ , respectively.

### 2.8. UV difference spectroscopy

HP8453UV-vis was used for all UV absorbance data collection. Each decalcified protein was diluted to 10 mM Hepes, pH 7.4 and the protein concentration determined spectroscopically. Aliquots of  $\text{Ca}^{2+}$  equivalent to the total Cen or G115W present were added individually up to 4 equiv of  $\text{Ca}^{2+}/\text{Cen}$  or  $\text{Ca}^{2+}/\text{G115W}$ .

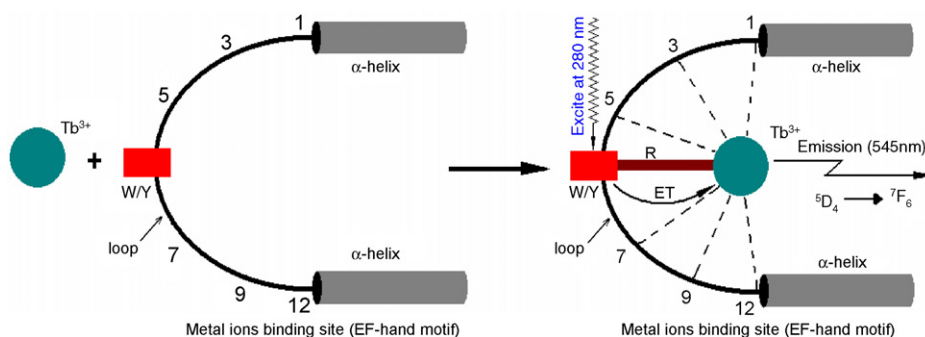
### 2.9. Resonance light scattering measurements

Solution turbidity changes induced by the addition of metal ions were monitored by the change of fluorescence at wavelengths 330 nm of the protein samples in quartz cells of 1-cm optical path at 25 °C.

## 3. Results and discussion

### 3.1. Binding of $\text{Tb}^{3+}$ to Cen or G115W by aromatic residue-sensitized luminescence

There have many reports on the binding of  $\text{Tb}^{3+}$  to CaM by monitoring the sensitized emission of  $\text{Tb}^{3+}$  at 545 nm as the protein is irradiated at 280 nm in the tyrosine absorption band [22–26], can be seen in Scheme 1. We carried out this experiment as shown in Fig. 2. The first 2 equiv. of added  $\text{Tb}^{3+}$  appear to bind quantitatively, but with minimal sensitization. As additional equivalents of  $\text{Tb}^{3+}$  are added, considerable sensitized emission is observed, which can be seen in Fig. 3A curve a. When the same titration was carried out in the presence of  $\text{Ca}^{2+}$  (present at 10 times the Cen concentration), the first 2 equiv. of  $\text{Tb}^{3+}$  again appear to bind quantitatively (Fig. 3A curve b). They are sensitized to a slightly lesser degree, perhaps due to the fact that the protein is in a different conformation owing to occupancy of the remaining binding sites by  $\text{Ca}^{2+}$ . Upon addition of  $\text{Tb}^{3+}$  beyond 2 equiv. in the presence of  $\text{Ca}^{2+}$ , the sensitized emission rises somewhat more slowly owing to a competition for the two weaker sites between the two types of ion. The final level of sensitized  $\text{Tb}^{3+}$  luminescence is the same for the titration in the absence of  $\text{Ca}^{2+}$ , proving full occupancy of the four binding sites by  $\text{Tb}^{3+}$  in the presence of this level of  $\text{Ca}^{2+}$ . The sensitized emission arises from a nonradiative energy transfer between the three tyrosine residues (the fourth located at the end of protein) and the bound  $\text{Tb}^{3+}$  ions. The titration curves thus imply that the



Scheme 1. Energy transfer sketch map from aromatic amino acid (W or Y) to  $Tb^{3+}$ . ET is energy transfer.  $R$  represents the distance between the donor (W or Y) and the acceptor ( $Tb^{3+}$ ), dashed represent the coordinated environment of  $Tb^{3+}$ .

tight binding sites, which are only feebly sensitized, are remote from the three tyrosine moieties in the molecule. Since the latter occur at positions 46, 72 and 79 of the polypeptide sequence in calcium binding N-terminal domain of the protein, the tight sites initially occupied by  $Tb^{3+}$  are sites III and IV. In the same conditions, the mutant was monitored by  $Tb^{3+}$  sensitized spectra (Fig. 3B). The results shows that the first equivalent of  $Tb^{3+}$  exhibits little sensitized luminescence, the second equivalent is sensitized to the large extent, and the third and fourth equivalent are, different from the second equivalent, sensitized to a more large extent. Through tryptophan (Trp) as a probe, we can have a conclusion that sites IV and III bind  $Tb^{3+}$  by the different affinity, with site IV higher than site III, in accord with the result reported previously [27].

In the canonical EF-hand, the loop contains three calcium-binding aspartic acid residues, which form the Dx Dx DG sequence motif, and is flanked by two  $\alpha$ -helices [28]. As shown in Table 1, site IV has six charged ligand residues and meet all of the criteria for classification as standard EF-hands. In addition, a lysine residue at positions 2 of the loop provide the stability required for proper  $Ca^{2+}$  geometry by neutralizing the repulsion among ligand residues 1, 3. In contrast, loop III lacks the the

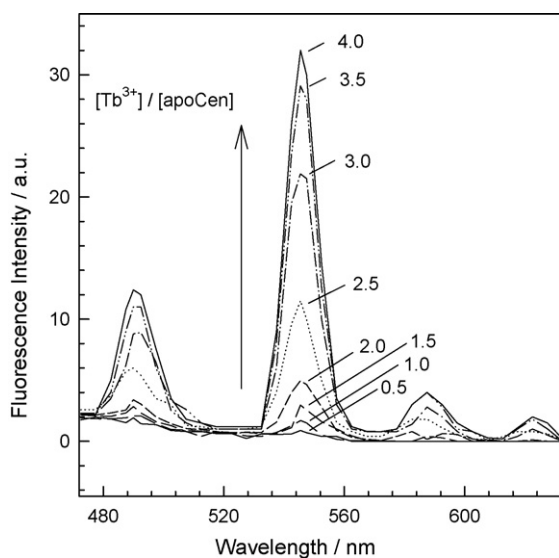
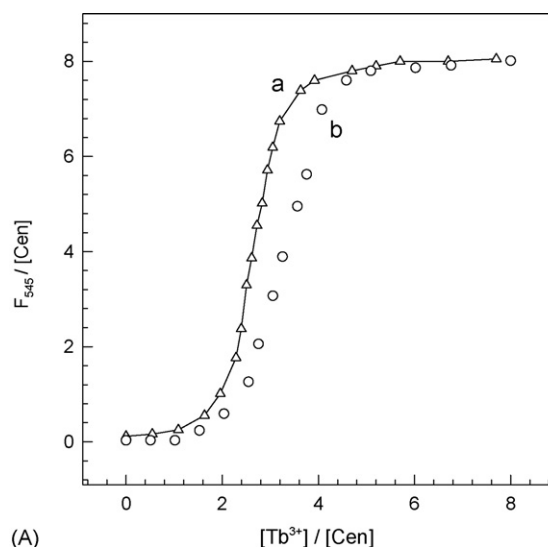
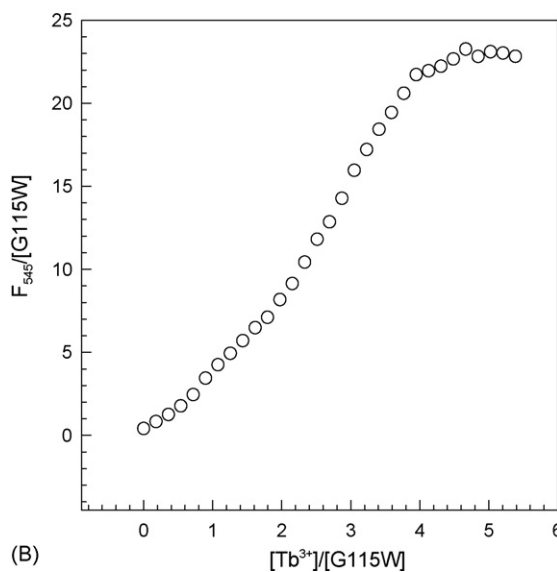


Fig. 2. Tyrosine-sensitized fluorescence spectra of  $Tb^{3+}$  bound to apoCen at metal to protein ratios of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0. The concentration of protein is  $5.48 \mu M$ .



(A)



(B)

Fig. 3. Titration curve for the addition of  $Tb^{3+}$  to proteins by measuring the fluorescence intensity at 545 nm in 10 mM Hepes, pH 7.4,  $25^\circ C$ . (A) The concentration of Cen is  $5.48 \mu M$ —a: apoCen, b: Cen; (B) the concentration of G115W is  $9.75 \mu M$ .

Table 1  
Comparison of the amino acid sequence of the four Ca<sup>2+</sup>-binding sites in Cen

MIKKPEFGLMQPPKRVROE																															
E-helix			Ca <sup>2+</sup> -loop									F-helix																			
	1	2	3	4	5	6	7	8	9	10	11	12																			
28					EF-hand I								56																		
ELSEEQKQ	E	I	K	E	A	F	D	L	F	<b>D</b>	<b>T</b>	<b>N</b>	<b>K</b>	<b>T</b>	<b>G</b>	<b>S</b>	<b>I</b>	<b>D</b>	<b>Y</b>	<b>H</b>	<b>E</b>	L	K	V	A	M	R	A	L		
64					EF-hand II								92																		
GFDVKKPE	E	I	E	L	M	<b>N</b>	<b>E</b>	<b>D</b>	<b>R</b>	<b>E</b>	<b>G</b>	<b>N</b>	<b>G</b>	<b>Y</b>	<b>I</b>	<b>G</b>	<b>F</b>	<b>D</b>	<b>D</b>	F	L	D	I	M	T	E	K				
101					EF-hand III								129																		
IKNRDPVE	E	I	L	K	A	F	K	V	<b>D</b>	<b>E</b>	<b>D</b>	<b>N</b>	<b>S</b>	<b>G</b>	<b>K</b>	<b>I</b>	<b>S</b>	<b>L</b>	<b>R</b>	<b>N</b>	L	K	R	V	A	K	E	L			
137					EF-hand IV								165																		
GENLSDD	E	L	Q	A	M	I	D	E	<b>D</b>	<b>K</b>	<b>D</b>	<b>Q</b>	<b>D</b>	<b>G</b>	<b>E</b>	<b>I</b>	<b>S</b>	<b>E</b>	<b>Q</b>	<b>E</b>	F	L	N	I	M	K	Q	T	S	I	Y

Aromatic amino acid (Y) display in italic font and having underline.

highly conserved Glu at position 12, which have been proved to be very important by site-directed mutagenesis experiments [29,30]. So the relative affinity of Tb<sup>3+</sup> in site III should be lower than that of site I, as it differs from our experiment result. One reasonable explanation is positive cooperativity existing between sites III and IV. This comparative sequence analysis strongly suggests that the two higher affinity sites in Cen are located in the C-terminal domain and the two lower affinity sites in the N-terminal domain and the relative affinity of site IV is greater than site III.

Our relative Tb<sup>3+</sup> affinities agree with the values predicted from the acid-pair hypothesis of Reid and Hodges [31]. They hypothesized that the relative Ca<sup>2+</sup>-binding affinity of EF-hand motifs depends on the number and arrangement of the charged ligand residues in the EF-loop. According to the acid-pair hypothesis, the relative Ca<sup>2+</sup>-binding affinity of these two sites in C-domain is IV > III, is also accord with a charge-ligand-balanced model proposed by Ye et al. [13]. In their model, both the number of negatively charged ligand residues (at loop positions 1, 3, 5, 7, 9, and 12) and the balanced electrostatic

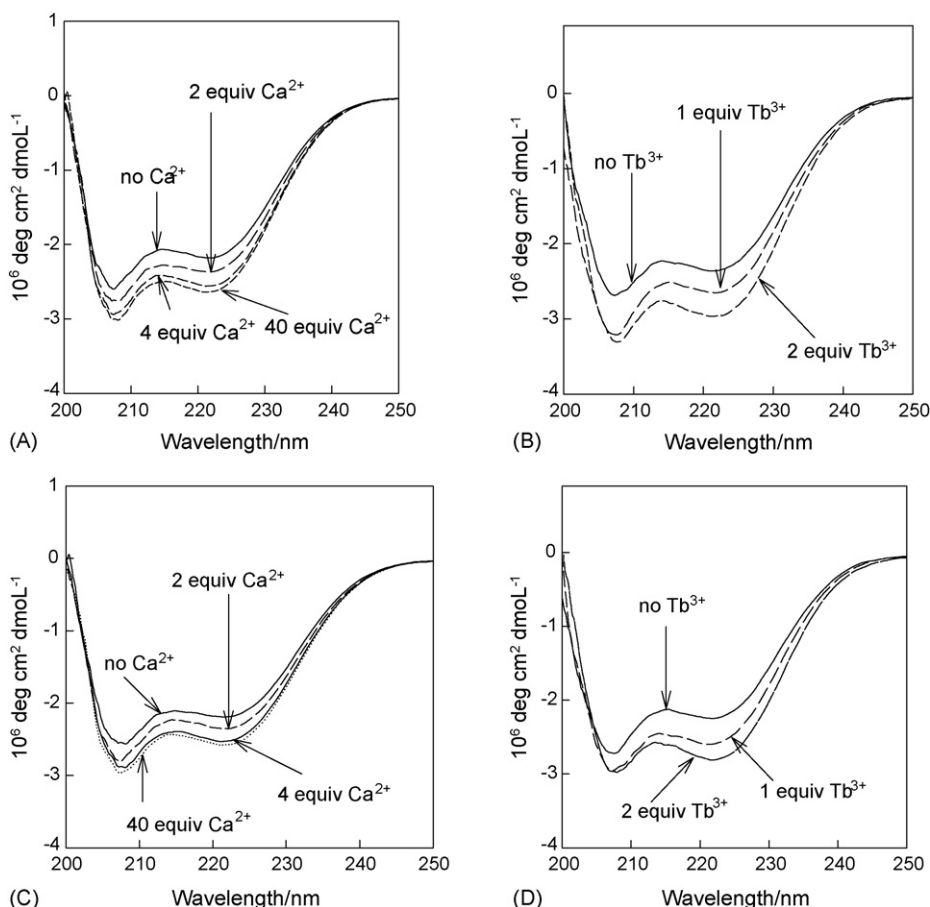


Fig. 4. Far-UV light CD spectra of Cen (A and B) and G115W (C and D) in the presence various levels of Ca<sup>2+</sup> or Tb<sup>3+</sup>. A and B samples contained 10.2 μM Cen, C and D samples contained 10.23 μM G115W, respectively. The experimental was carried out in 10 mM Hepes, pH 7.4, 25 °C.

dentate–dentate repulsion by the adjacent charged residues determine the relative  $\text{Ca}^{2+}$ -binding affinities.

### 3.2. Analysis of conformational changes from circular dichroism spectroscopy

Circular dichroism was used to characterize the distribution of secondary structural elements the difference in the conformation between Cen and G115W, and determine whether any changes occurred upon binding  $\text{Ca}^{2+}$  and/or  $\text{Tb}^{3+}$ . The far-UV light CD spectra of Cen at various  $[\text{Ca}^{2+}]/[\text{Cen}]$  ratios are highly similar in shape, with two minima at 208 and 222 nm, typical of a protein rich in  $\alpha$ -helix (Fig. 4A). An increase in molar ellipticity (222 nm) is observed upon the addition of  $\text{Ca}^{2+}$ . Fig. 4B is the far-UV light CD spectra of Cen in the presence of  $\text{Tb}^{3+}$ . Owing to having obvious polymerization, spectra were only obtained in the range of  $[\text{Tb}^{3+}]/[\text{Cen}]$  ratio of 0–2. As can be seen in Fig. 4C and D, the conformational change of G115W induced by  $\text{Ca}^{2+}/\text{Tb}^{3+}$  is similar to that of Cen. Compared the CD spectra between Cen (Fig. 4A) and G115W (Fig. 4C), the following conclusions can be drawn that the secondary conformation of Cen and G115W is very similar, and further the substitution of glycine to tryptophan at position 115 have no obvious effect on the conformation of Cen. This is agreement with the results obtained by following UV difference spectra.

### 3.3. $\text{Tb}^{3+}$ binding induced changes in the conformation of Cen and G115W

Calcium induced exposure of hydrophobic surfaces has been shown to be a key step in signal transduction by EF-hand  $\text{Ca}^{2+}$ -sensor proteins. A useful probe of such conformational changes is the hydrophobic fluorophore TNS [32,33], because its fluorescence is altered when it binds to hydrophobic patches on the accessible surface of proteins. Fig. 5 shows the fluorescence intensity change of TNS at 440 nm in the presence of the centrin

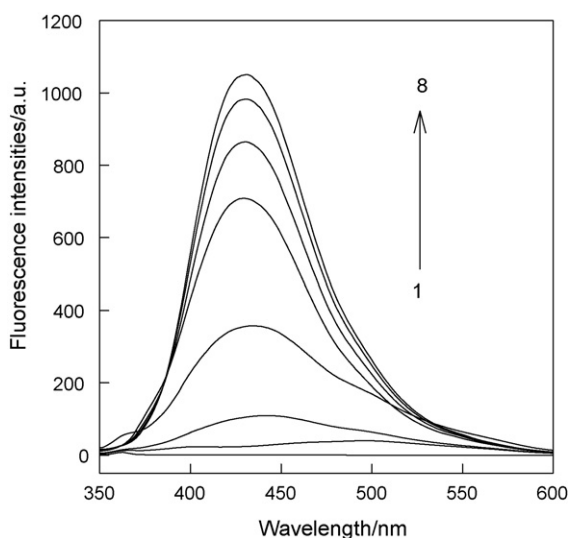


Fig. 5. Fluorescence spectra of the TNS with the addition of  $\text{Tb}^{3+}$  in the presence of Cen.

using  $\text{Tb}^{3+}$  as titrator. As seen from Fig. 5, the addition of  $\text{Tb}^{3+}$  leads to increases in the fluorescence intensity and concomitant blue shifts in the maximum wavelength of emission, indicating the exposure of hydrophobic patches on the molecular surface of centrin. It is of note that saturation of the TNS fluorescence change is reached at around 4 molar equiv. of  $\text{Tb}^{3+}$ , suggesting the TNS fluorescence change is proportional to the hydrophobic surface exposure of centrin induced by  $\text{Tb}^{3+}$  binding, and the exposure extent of hydrophobic surface is possible responsible for the various biological function in the sensing and modulating of  $\text{Ca}^{2+}$  signals. The titration curve was obtained through TNS fluorescence intensity at 440 nm as function of total equivalent of  $\text{Tb}^{3+}$  added to Cen, shown as Fig. 6A. Fig. 6A indicated that sites I and II contribute largely more than the other two sites to overall conformation change, and the difference between the III

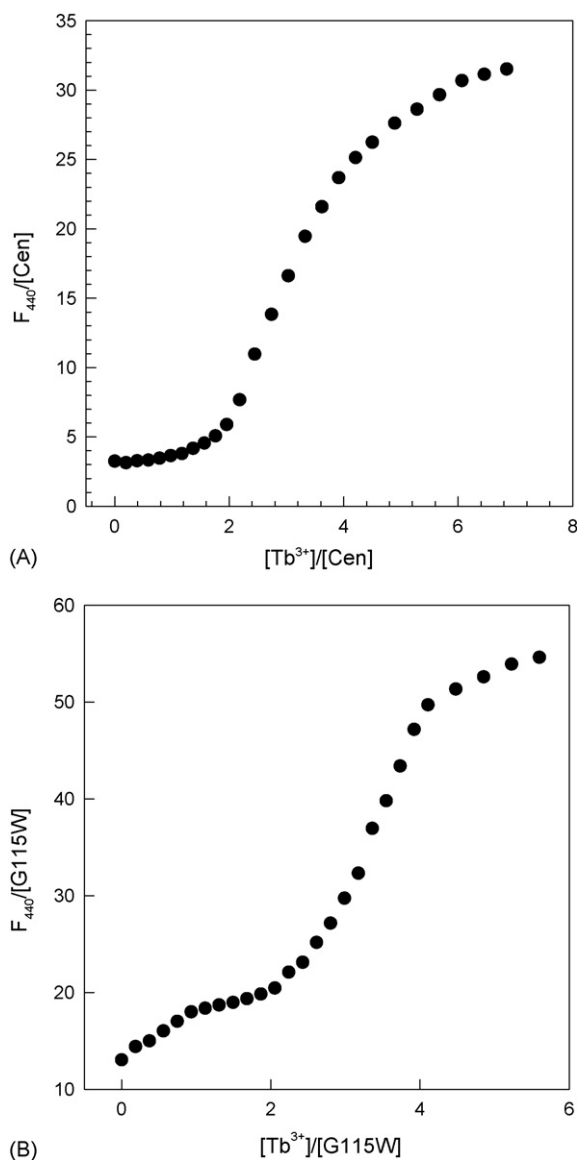


Fig. 6. Titration curve of the addition  $\text{Tb}^{3+}$  to centrin (A) and the mutant G115W (B) in the presence of the TNS by measuring the fluorescence intensity at 440 nm. (A) The concentration of Cen is 19.38  $\mu\text{M}$  containing 21.12  $\mu\text{M}$  TNS; (B) the concentration of G115W is 91.98  $\mu\text{M}$  containing 19.12  $\mu\text{M}$  TNS.

and IV site is little, in other words, N-terminal domain have a larger conformation change than C-terminal domain when bind to metal ion (such as  $Tb^{3+}$  and  $Ca^{2+}$ ).

As can be seen in Fig. 6B, with the  $Tb^{3+}$  addition, the fluorescence intensity change of the mutant is similar to that of Cen, the major difference being that in G115W for the sites III and IV in the C-terminal domain of Cen, the TNS fluorescence intensity increase with addition of 1 equiv.  $Tb^{3+}$ , and do almost not change when 2 equiv.  $Tb^{3+}$  are added. Compared titration curve of G115W with Cen, we can draw the conclusion that Cen exist site–site interaction, according to the mutant in the site III, the property of metal ion binding in the site IV is affected in some extent. The change of “closed” conformation to “open” conformation in the C-terminal domain in response to binding of  $Tb^{3+}$ , is mostly owing to 1 equiv.  $Tb^{3+}$  binding to the site IV, indicating the site IV has an important function in the process of C-terminal domain realizing itself biological function.

The different function of N-terminal domain and C-terminal domain can be attributed to the distinct extent of hydrophobic surface exposure induced by  $Ca^{2+}$  binding. The interaction of *Chlamydomonas reinhardtii* centrin with Kar1p is mediated by the C-terminal domain, which leaves the N-terminal domain of centrin to function as a  $Ca^{2+}$  sensor that interacts with other target proteins [27]. In skeletal muscle, the C-terminal domain of troponin C is constitutively bound to the N-terminal domain of troponin I, whereas the N-terminal domain of troponin C interacts with the C-terminal domain of troponin I in a calcium-dependent manner [34,35]. Exposure of hydrophobic surface is essential to the different functions between N-terminal and C-terminal domain. So using TNS as a probe, the basis of that the N-terminal domain and C-terminal domain play distinct biological roles can be obtained.

### 3.4. UV difference spectroscopy studies

Cen contains no cysteine or tryptophan and has four tyrosine residues which lies at position 46, 72, 79 and 168, Tyr-46 in the “loop” of  $Ca^{2+}$ -binding site I and Tyr-72 and Tyr-79 are close to “loop” and in the “loop” of  $Ca^{2+}$ -binding site II, respectively. Tyr-168, whose environment is not much influenced by  $Ca^{2+}$  binding [33], is the last residue of Cen.  $Ca^{2+}$ -induced alterations to the environment of tyrosine can be monitored by UV difference spectroscopy (see Figs. 7A and 8A for wild-type UV spectra and difference spectra).  $Ca^{2+}$ -induced changes in the phenylalanine absorption region of the Cen UV spectrum are also seen, but these are difficult to interpret since the 10 phenylalanine residues of the protein are found throughout its various domains. Fig. 7A shows the overall change to the UV spectrum of Cen when excess  $Ca^{2+}$  is added to the  $Ca^{2+}$ -free protein. The major change is an apparent  $Ca^{2+}$ -induced decrease in absorbance at the tyrosine peak and shoulder (279 and 286 nm, respectively). In Fig. 8 the UV difference spectra upon sequential addition of several equivalents of  $Ca^{2+}$  to the wild-type protein and the mutant G115W. This displays the decreased tyrosine absorbance as negative peaks centered around 279 and 286 nm and the peak at 286 nm is not obvious, which is shaded by the strong peak at around 279 nm. However, the profile of the tyrosine UV differ-

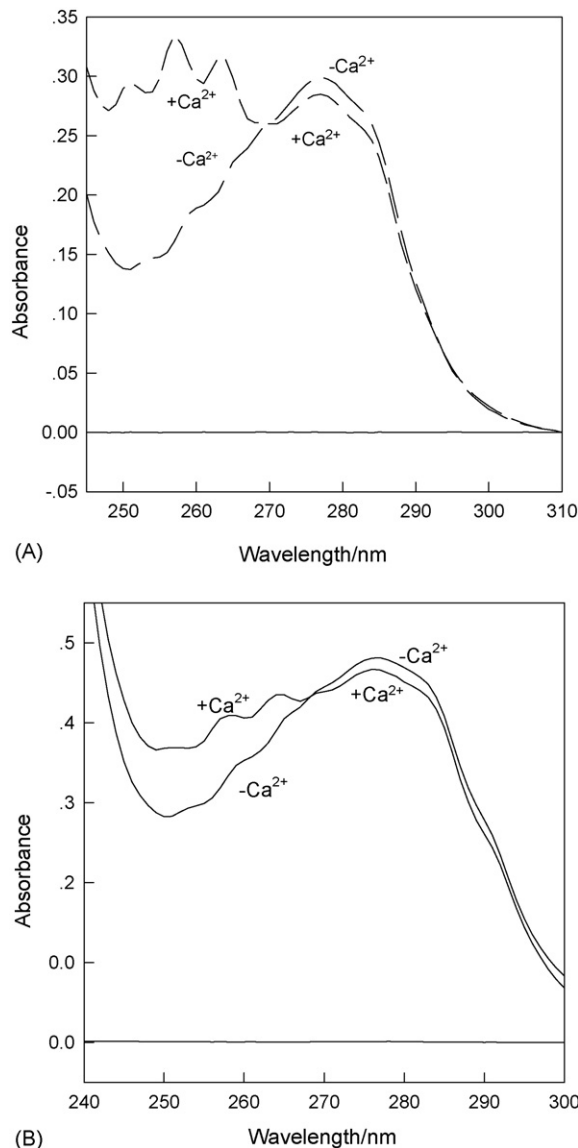


Fig. 7. UV spectra of the Cen (A) and mutant G115W (B) in 10 mM HEPES, pH 7.4, 25 °C. + $Ca^{2+}$ , spectrum of protein at 119  $\mu$ M (A) and 90.96  $\mu$ M (B), 1 mM  $CaCl_2$ ; - $Ca^{2+}$ , spectrum of protein at the same concentration, 1 mM EDTA.

ence spectrum seen here (265–290 nm), which is characterized by the greater decreases at 279 than 286 nm (Fig. 7A), is unusual when compared with other proteins. The unusual behavior of the calmodulin UV difference spectrum has been seen previously for mammalian calmodulin and presumably reflects an environment for Tyr-138 producing unique electronic interactions for this residue [36,37]. A decrease in tyrosine absorbance in proteins is typically associated with the exposure of tyrosine to a more polar environment. This unusual behavior had also been described previously by Maune et al. [29]. On the other hand, there are several positive peaks in the region of phenylalanine absorbance, showed that phenylalanine residues are exposed to a more hydrophobic environment induced by  $Ca^{2+}$  binding.

As can be seen in Fig. 8A, for the wild-type protein, the major changes to the tyrosine UV difference peaks are largely from the addition of a second two equivalents of  $Ca^{2+}$ . The intensity still

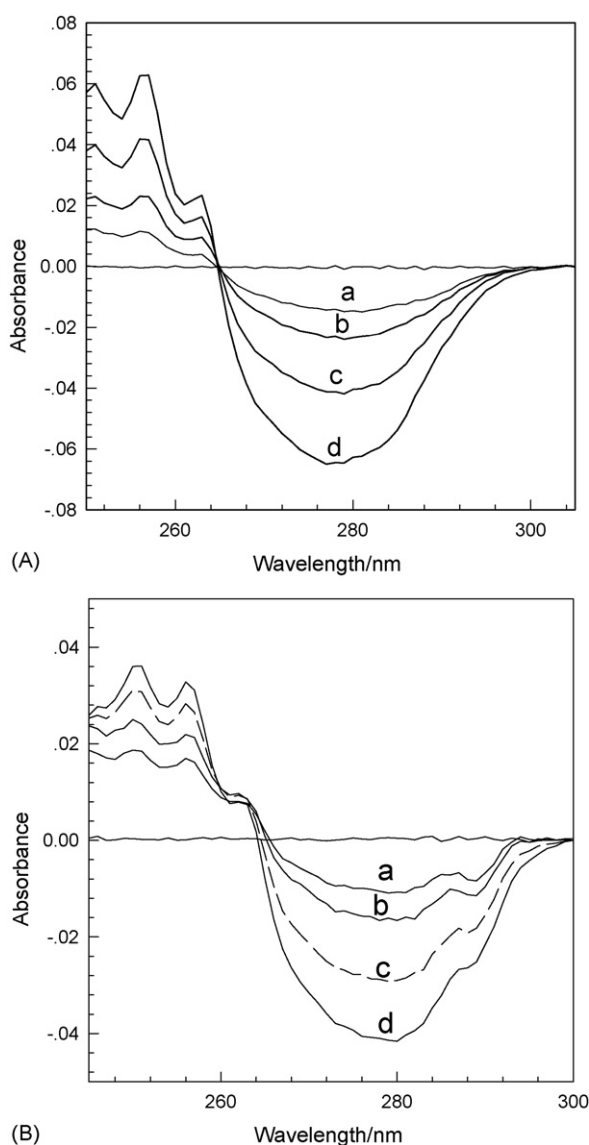


Fig. 8. UV difference spectra of Cen (A) and G115W (B) in 10 mM Hepes, pH 7.4. Four individual equivalent of  $\text{Ca}^{2+}$  were added sequentially to aliquots of each protein, a, b, c, d are represent 1, 2, 3 or 4 equiv. of  $\text{Ca}^{2+}$ , respectively. (A) The concentration of Cen is 119  $\mu\text{M}$ ; (B) the concentration of G115W is 90.96  $\mu\text{M}$ . The difference spectra still increase even after 10 equiv. (data not shown).

increases until the 10 equiv.  $\text{Ca}^{2+}$  was added. For the mutant (Fig. 8B), the changes to these peaks around 280 nm are very similar to those seen for the wild-type protein, the major difference being that there is a small peak at around 290 nm, which is owing to the change of Trp environment. These findings indicate that mutation of binding site III has little effect on the conformational change monitored through Tyr. So using the Trp as a structural probe is suitable way.

### 3.5. Resonance light scattering spectra

In this light scattering experiment, we measured the fluorescence intensity change of Cen and the mutant at 330 nm with the addition of  $\text{Tb}^{3+}$ . As can be seen from Fig. 9A, The first 2 equiv.

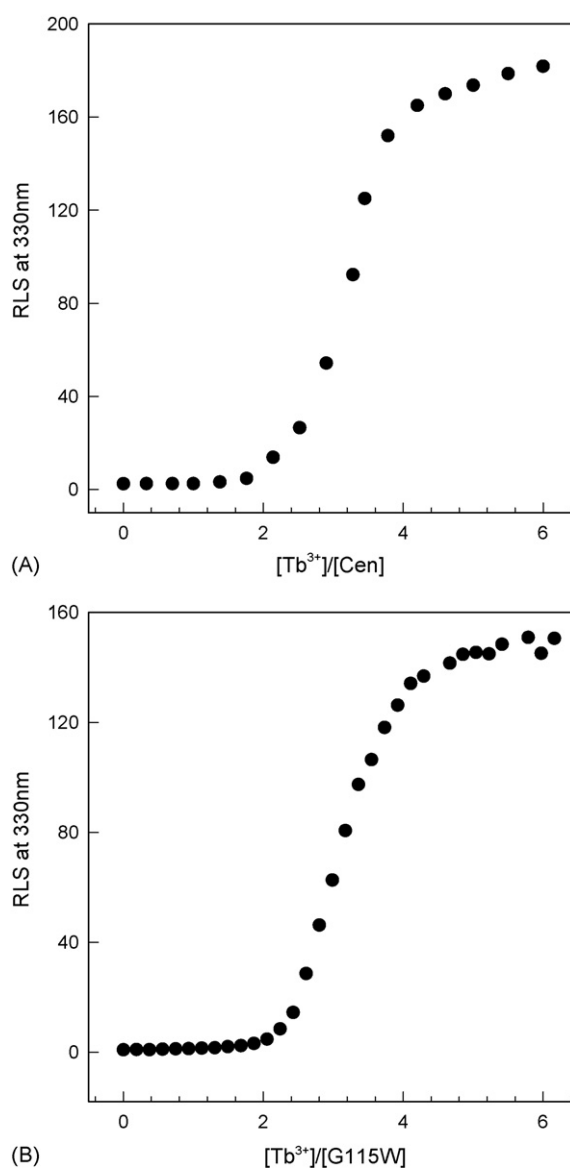


Fig. 9. Resonance light scattering of Cen (A) and mutant G115W (B) at 330 nm with the addition of  $\text{Tb}^{3+}$ .

of added  $\text{Tb}^{3+}$  have little effect to the polymerization. Until the another 2 equiv. were added, the fluorescence intensity appear to obviously increase, indicating N-terminal has a large contribution to the polymerization. The result can be testified by simple “baked eye”. The protein solution had no obvious sedimentation until the 3 equiv.  $\text{Tb}^{3+}$  was added. If the EDTA solution was added, the sedimentation was disappear, showing the polymerization is reversible, and G115W (Fig. 9B) is similar to Cen. The extent of sedimentation is relative with the concentration of protein.

Wiech et al. showed that centrins from yeast, algae *Scherffelia dubia* centrin, or humans may form multimers in the presence of  $\text{Ca}^{2+}$ , in clear contrast with the yeast calmodulin [38]. All caltractins contain a positively charged amino terminal sequence of variable length [10]. With addition of  $\text{Tb}^{3+}$ , centrin undergo a conformation change from “closed” to “open”, meanwhile, the negative charged amino acids (D,E), which possible assem-



ble with the positively charged amino acid in the N-terminal of another subunit in the Cen, are exposed to protein solution. This assembly possible function as a initial factor of the polymerization. Evolution of the complexes toward larger polymers requires the participation of a highly basic N-terminal segment including the first 25 residues [39]. Tourbez et al. [27] have testified an increased monovalent concentration (>50 mM) inhibits the polymerization of human centrin 2. It provide some evidences that the polymerization induced by Tb<sup>3+</sup> binding is owing to the reaction between the negative charge amino acids of the C-terminal domain and positively charged amino located in N-terminal domain, because this reaction is affected by the increase of the ion strength.

In summary, we have shown that there is cooperativity between the sites IV and III, which are higher affinity sites and the relatively affinity of site IV is greater than that of site III using fluorescence titration experiment, Tb<sup>3+</sup> and tryptophan as probes. In addition, through hydrophobic probe TNS, the contribution extents of hydrophobic surface exposure are investigated from the different sites, which is possible responsible for the distinct biological function in the Ca<sup>2+</sup> signal sensing and reacting the target peptide in the cell. In the end, we have explored some properties of the Cen polymerization, indicating the N-terminal plays a key role in the polymerization.

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