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Effect of Tb(III) on the unfolding of *ciliate Euplotes octocarinatus* centrin induced by guanidine hydrochloride☆

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

[a] *Institute of Molecular Science, Shanxi University, Taiyuan 030006, China*
[b] *Department of Pharmacy, Shanxi Medical University, Taiyuan 030001, China*

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ABSTRACT

To understand the unfolding of *ciliate Euplotes octocarinatus* centrin (EoCen), the glycine positioned at 115, the sixth residue of the loop of the protein's third EF-hand, was mutated into tryptophan (Trp). Intrinsic fluorescence and Tb(III) binding properties of wild type EoCen and G115W mutant were monitored by fluorescence spectra in 10 mmol/L Hepes. The emission maximum of EoCen was 306 nm and mutation had no impact on the Tb(III) binding properties. The properties of G115W were investigated by fluorescence, far-UV circular dichroism (CD) spectra and fluorescence decays in the absence or in the presence of 6 mol/L guanidine hydrochloride (GdnHCl). For the increase in polarity of microenvironment around Trp residue, the emission maximum of apoG115W at 343 nm is shifted to 359 nm in 6 mol/L GdnHCl. Also the secondary structure is lost nearly and fluorescence lifetime decreases in 6 mol/L GdnHCl. The unfolding of G115W induced by GdnHCl was assessed by using the model of structural element. The unfolding of proteins is a sequential reaction, namely two-transition, three-state process. The first transition belongs to the unfolding of the C-terminal domain, and the second transition is assigned to the unfolding of the N-terminal domain. The $\Delta\langle\Delta G^0_{total}(H_2O)\rangle$ was used to determine the effect of Tb(III) on the stability of apoprotein. The $\langle\Delta G^0_{total}(H_2O)\rangle$ for Tb_2-G115W has a less increase of 0.68 kJ/mol compared with apoG115W, proving Tb(III) situated at C-terminal has negligible impact on the stability of protein. Whereas the $\langle\Delta G^0_{total}(H_2O)\rangle$ for Tb_4-G115W has a rise of 1.29 kJ/mol compared with Tb_2-G115W, manifesting Tb(III) located at low affinity sites has considerable influence on protein stability, mainly stabilizing the N-terminal domain.

1. Introduction

Centrins are small (~20 kDa) acid calcium binding proteins from calmodulin (CaM)[1] superfamily, well-conserved in the eukaryote kingdom from algal and yeast to humans. Also centrins share approximately 50% sequence identity with the well-studied EF-hand protein CaM. Like CaM, centrins contain two relatively independent global domains connected by a flexible linker, providing a dumbbell-like shape, each one including two helix-loop-helix motifs, the so-called EF-hand. The N-terminal domain of centrins contains a highly variable and unordered segment of 20–25 residues and exhibits more variable calcium binding properties relative to the C-terminal domain. Each molecule possesses four latent calcium binding sites (I–IV), but because of amino acid substitutions in the binding loop or in the adjacent helices, the affinity and specificity for calcium ion may alter from one species to another.

Ciliate *Euplotes octocarinatus* centrin (EoCen), a protein of 168 residues, was first reported by our group[2] (GenBank accession number: Y18899), which is cloned from *Euplotes octocarinatus*. Due to the similar coordination chemical properties and ionic radii of calcium ion and lanthanide ion, rare earth ions, nonbiogenic metal species, can be widely used to probe metal binding property of apoprotein[3,4] by substituting native cofactor, which are silent in spectroscopic studies.[5] In protein the tryptophan residue is often regarded as an intrinsic fluorescent detector to monitor protein structure change, because tryptophan fluorescence is strongly affected by its physica-chemical conditions.[6] There is no tryptophan in EoCen, hence the glycine positioned at 115, the sixth

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

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residue of the loop of the protein's the third EF-hand, was mutated into tryptophan (Trp).

Studying the mechanisms of the folding and the unfolding of the proteins remains one of the important issues in molecular biology.[7,8] Metal ions serve as cofactor and play a regular role in protein's structure and functions.[9] Therefore it is necessary to explore the impact of metal ions on the proteins. Chemical denaturation is an applicable way to measure and compare structural stability of proteins.[10] The distinction in the slope, transition midpoints, and the whole shape of the unfolding curves reflected structural differences of proteins. The present work described the interaction of EoCen mutant G115W with guanidine hydrochloride (GdnHCl), using fluorescence spectroscopy of intrinsic Trp, far-UV circular dichroism (CD) and fluorescence lifetime. Meanwhile the effect of Tb (III) on the structural change and stability of EoCen was also investigated.

2. Experimental

2.1. Protein expression and purification

Expression and purification of the *Euplotes octocarinatus* centrin and mutant G115W were carried out according to our previous publications.[2] After purification, the proteins were kept at −20 °C, and the stock protein solutions were preserved in 10 mmol/L Hepes, pH 7.4.

2.2. Aromatic residue sensitized Tb^{3+} emission

Aromatic residue-sensitized Tb^{3+} emission was performed with apoprotein concentration of 6 μmol/L in 10 mmol/L Hepes pH 7.4, 25 °C. Transferring 2 mL above solution into 1 cm quartz cell, Tb^{3+} stock solution was added directly to apoprotein. The mixture was shaken thoroughly by hand, and then equilibrated for 3 min in order to make Tb^{3+} bind to protein completely. To avoid secondary Raleigh scattering, Tb^{3+} emission fluorescence spectra were acquired from 470 to 570 nm with excitation at 290 nm for G115W and 280 nm for EoCen. Both slit widths of excitation and emission were 5 nm. To correct the dilution effect, the fluorescence intensity was converted to molar fluorescence intensity by dividing the fluorescence intensity via the analytical concentration of protein in the titration curve. The titration curve described the normalized fluorescence intensity values at 545 nm vs a function of ratio of Tb^{3+} to protein.

2.3. Fluorescence spectroscopy

The experiments were performed on a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, France). The excitation wavelengths were 295 nm for G115W and 280 nm for EoCen, respectively. Fluorescence emission spectra were recorded from 310 to 560 nm, with all slit widths set to 5 nm, using 1 cm quartz cuvettes.

2.4. Far-UV CD measurements

CD experiments were performed on a Chirascan spectrometer (Applied Photophysics Ltd. UK) continuously purged by N_2 and equipped with a temperature-control system. Far-UV spectra were recorded between 190 and 250 nm at 25 °C using 1 mm quartz cells. Spectra were collected as an average of three scans with a step size of 0.2 nm and a bandwidth of 1 nm. Samples were dissolved in 10 mmol/L Hepes (pH 7.4) at a concentration of G115W of 8 μmol/L.

2.5. Fluorescence lifetime measurement

Fluorescence lifetimes were collected by time-correlated single-photon counting technique on an Edinburgh Analytical Instrument type nF-900 fluorometer. The excitation source was a picosecond pulsed laser diode. Ludox HS-30 scattering solution was used to collect the instrument's response. The fluorescence decay of tryptophan was analyzed using a sum of exponentials:

$$I(t) = \sum A_i \exp(-t/\tau_i) \quad (1)$$

where A_i and τ_i are the amplitude and lifetime, respectively, of the ith component. $I(t)$ was convoluted with the experimental response and then compared with the experimental data by nonlinear least-squares methods. The average fluorescence lifetime was calculated by the following equation:

$$\tau_0 = \frac{\sum \tau_i^2 A_i}{\sum \tau_i A_i} \quad (2)$$

2.6. Chemical denaturation

GdnHCl-induced unfolding of apoG115W and holo-G115W were performed, in 10 mmol/L Hepes, pH 7.4, 25 °C. In each denaturation experiment, every sample was titrated with GdnHCl from 0 to 6 mol/L, at a protein concentration of 4 μmol/L. All samples were incubated for 30 min before emission measurements. There was no time dependence in the unfolding reaction between 30 min and 24 h.

2.7. Analysis of denaturation data

The curves (the fluorescence intensity ratio of 400 nm to 340 nm vs GdnHCl concentration) were normalized to provide the fraction of unfolded molecules as a function of the denaturant concentration. Three-state unfolding curves were analyzed using the new model proposed by our group to obtain the values of the free energy of the structural element unfolding in water $\langle \Delta G^0_{\text{element}}(H_2O) \rangle$ and the midpoint of transition $D_{1/2}$.[11]

The model was used to calculate the free energy change of protein unfolding. In this model, it is considered that proteins are composed of structural elements. The unfolding of a structural element obeys a two-state mechanism and the free energy change of the element can be gotten by a linear extrapolation method. The free energy change of protein is the sum of free energy changes of structural element.

3. Results and discussion

3.1. Intrinsic fluorescence and Tb^{3+} binding property

EoCen consists of no cysteine or tryptophan and has four tyrosine residues which located at position 46, 72, 79 and 168. Tyr-46 lies in the 1st loop and Tyr-72 and Tyr-79 are close to and in the 2nd loop of Ca^{2+}-binding site, respectively. Tyr-168 is at the tail end of protein, which is outside the 4th Ca^{2+}-binding site. As shown in Fig. 1(a), EoCen showed intrinsic fluorescence, and the emission maximum was 306 nm. Whereas for G115W, in which Trp residue was positioned at 6th of the 3th loop region in the C-terminal domain, the emission maximum was shifted to 343 nm, as characteristic of Trp residue. Ca^{2+} is the native cation of EoCen, however it is spectroscopically silent. So we used Tb^{3+} to probe the binding ability of metal ions to protein. For EoCen, the excitation wavelength was set at 280 nm. Sensitized emission of bound Tb^{3+}