八肋游仆虫中心蛋白 N-端半分子与 Tb^{3+},Ca^{2+}的结合

任列香 赵亚琴 丰九英 贺晓静 梁爱华 杨斌盛*
(山西大学分子科学研究所,化学生物学与分子工程教育部重点实验室,太原 030006)

摘要: 用分子生物学方法表达、纯化了游仆虫中心蛋白及 N-端半分子,用铽荧光探针法、离子竞争法研究了 pH 7.4,0.01 mol·L^{-1} Hepes 条件下中心蛋白与铽、钙的结合性质。结果表明中心蛋白有 4 个铽结合部位,其中 2 个为高亲合结合部位、2 个为低亲合结合部位。具有 2 个低亲合结合部位的中心蛋白半分子与铽结合的条件常数是$(2.13\pm0.10)\times10^5$ L·mol^{-1},与钙结合的条件常数是$(7.52\pm0.02)\times10^2$ L·mol^{-1}。

关键词: 中心蛋白; 荧光; 铽离子; 钙离子
中图分类号: O614.23; O614.33; Q51 **文献标识码**: A **文章编号**: 1001-4861(2006)01-0087-04

Terbium- and Calcium-binding Properties of N-terminal Domain of *Euplotes* Centrin

REN Lie-Xiang ZHAO Ya-Qin FENG Jiu-Ying HE Xiao-Jing LIANG Ai-Hua YANG Bin-Sheng*
(*Institute of Molecular Science, Key for Laboratory Chemical Biology and Molecular Engineering, Education of Ministry, Shanxi University, Taiyuan* 030006)

Abstract: *Euplotes* centrins (EoCens) including full-length protein (apoEoCen) and semi-molecule centrin (*N*-apoEoCen) were expressed and purified by biological engineering. The binding of terbium (Tb^{3+}) to apoEoCen was monitored by fluorescence in 0.01 mol·L^{-1} *N*-2-hydro-xyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), at pH 7.4. It could be seen that the protein binds two Tb^{3+} with high affinity and two Tb^{3+} with low affinity. The reactions between *N*-apoEoCen and Tb^{3+} or Ca^{2+} were also studied by Tb^{3+} fluorescence probe and ionic competition in 0.01 mol·L^{-1} Hepes, at pH 7.4. On the basis of fluorescence titration curves, the conditional binding constant of Tb_2-*N*-EoCen was determined to be $(2.13 \pm 0.10) \times 10^5$ L·mol^{-1} and the relative binding constant of Ca_2-*N*-EoCen was calculated to be $(7.52 \pm 0.02) \times 10^2$ L·mol^{-1}.

Key words: *Euplotes* centrin; fluorescence; Tb^{3+}; Ca^{2+}

0 Introduction

Lanthanides have been known for their diversity in biological effect and widely applied in agriculture and medicine for the treatment of a series of diseases and diagnosis in magnetic resonance imaging[1,2]. It was suggested that the particulate- and protein-bound lanthanides entered cell by endocytosis, the anionic low-molecular-mass complexes, via anion channels, whereas free lanthanide ions were transported by ionophores, Na^+/Ca^{2+} exchange and self-facilitated diffusion[3,4]. Evidently, the interaction of lanthanides with intracellular proteins is very important.

Centrin, also known as "caltractin", was first identified in the green alga Tetraselmis striata. It is an acidic, 20-kDa calcium-binding protein, which belongs to the calmodulin superfamily of calcium-modulated proteins and closely related to calmodulin. Cen-

收稿日期:2005-07-08。收修改稿日期:2005-10-24。
国家自然科学基金(No.20371031)和山西省自然科学基金(No.20031017)资助项目。
*通讯联系人。E-mail:yangbs@sxu.edu.cn
第一作者:任列香,女,27 岁,硕士研究生;研究方向:生物无机化学。

trins play a fundamental role in centrosome duplication and contraction of centrin-based fiber systems[5]. Both centrin and calmodulin are comprised of two structurally independent globular domains connected by a flexile linker, and each domain contains two helix-loop-helix calcium binding motifs[6]. Like calmodulin, the helix-loop-helix motif in centrin is the most conserved feature in the protein[7]. As well known, the lanthanides, due to the similarity to Ca^{2+} in coordination chemistry, can compete with Ca^{2+} at calcium binding sites[8]. Since the fluorescence enhancement there have been a number of studies on Tb^{3+} binding to proteins[9,10], while there are few reports on the interaction of terbium with centrin. In this paper we report the Tb^{3+}- and Ca^{2+}- binding characteristics of *N*-terminal domain of Euplotes centrin (*N*-apoEoCen).

1 Experimental

1.1 Materials and instruments

N-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), Potassium dihydrogen phosphate, sodium hydrogen, metallic chlorides were all analytical reagents, obtained from Sigma-Aldrich, USA. Terbium oxide (Tb_4O_7) was 99.99%, purchased from Rare Earth Research Institute of Hunan, China.

Tryptone, yeast extract, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), sodium chloride and agar were obtained from Sangon Ltd. Glutathione Sepharose™ 4B (GST), Hitrap Q were purchased from Pharmacia Ltd.

A pair of primers, p1(5′-GCC GGA TCC ATA AAG AAG CCA GAA TTC-3′) and p2 (5′-GCC GTC GAC TTA TTC TAC AGG ATC TCT-3′), were synthesized by Biological company of Shanghai.

The instruments used are Hewlett Packard 8453 spectrophotometer, F-2500 fluorescence spectrophotometer, Hitachi 850 fluorescence spectrophotometer and pH meter.

1.2 Methods

1.2.1 Stock solution

Terbium stock solution (22.8 mmol·L^{-1}) was prepared by dissolving the terbium oxide (Tb_4O_7, ~0.853 3 g) in a small volume of concentrated hydrochloric acid. The solution was diluted in a 50.00 mL volumetric flask with deionized water. EDTA standardization of the terbium stock solution was performed in acetate buffer at pH 5.5. Xylenol orange was selected as the indicator. End point may be observed with change of color from pink to yellow. The calcium stock solution (1.0 mol·L^{-1}) was prepared by dissolving 5.551 0 g $CaCl_2$ in a small volume of distilled water. The solution was diluted into a 50.00 mL volumetric flask with deionized water.

1.2.2 Expression and purification

A pair of primers, p1(5′-GCC GGA TCC ATA AAG AAG CCA GAA TTC-3′) and p2 (5′-GCC GTC GAC TTA TTC TAC AGG ATC TCT-3′) with *BamH* Ⅰ and *Sal* Ⅰ restriction site were designed to obtain the expression recombinant plasmids pGEX-6P-1-N-Eocentrin. ApoEoCen and *N*-apoEoCen proteins were expressed and purified essentially as reported [11]. The concentration of *N*-apoEoCen was calculated from the absorbance at 276 nm based on an extinction coefficient of 4 350 L·mol^{-1}·cm^{-1} by UV.

1.2.3 Spectral measurements

UV spectra were measured from 230~400 nm with a Hewlett Packard 8453 spectrophotometer at room temperature. Fluorescence spectra were recorded on an F-2500 fluorescence spectrophotometer. Fluorescence intensities were measured with a Hitachi 850 fluorescence spectrophotometer. The solution of *N*-apoEoCen in 0.01 mol·L^{-1} Hepes at pH 7.4 was added to a dry fluorescence cuvette and titrated with Tb^{3+}. The excitation wavelength was 280 nm and emission wavelength was 545 nm with a filter of 390 nm. To correct for dilution during each titration, the fluorescence intensity at the maximum emission peak was converted to molar fluorescence intensity (F_M). Allowing for equilibrium time, spectra were recorded at 2-min intervals after the addition of Tb^{3+}. Using Tb^{3+} as fluorescence probe, Ca^{2+}- binding properties were explored from the competition between Ca^{2+} and Tb^{3+}.

In the experiments, all glasswares including cuvette were routinely immersed in 1.0 mol·L^{-1} HNO_3 and then rinsed with deionized water before use.

2 Results and discussion

2.1 Fluorescence of Tb^{3+} with apoEoCen

If there is an aromatic near-ultraviolet chromophore in the coordination sphere, the sensitization and enhancement of Tb^{3+} fluorescence via aromatic chromophore to Tb^{3+} radiationless energy-transfer processes will be possible to appear when the complex is irradiated in the near-ultraviolet absorption region of the aromatic moiety. Fig.1 shows the Tb^{3+} ion fluorescence titration spectra for Euplotes centrin (apoEoCen) in 0.01 mol·L^{-1} Hepes, pH 7.4, recorded in the range 470~630 nm under excitation at 280 nm. In the presence of apoEoCen (5.5 μmol·L^{-1}), the fluorescence of Tb^{3+} at 490 nm, 545 nm, 590 nm, 623 nm, the transition from $^5D_4$ to $^7F_6$, $^7F_5$, $^7F_4$, and $^7F_3$, respectively, is enhanced greatly, while the fluorescence of free Tb^{3+} (6.2 μmol·L^{-1}) can be neglected (curve a in Fig.1). It means that the binding of apoEoCen to Tb^{3+} and the energy-transfer from Tyr residues to the binding Tb^{3+} makes the fluorescence of Tb^{3+} enhanced. The plot of F_M, the fluorescence intensity of Tb^{3+} at 545 nm divided by the concentration of apoEoCen, versus $c_{Tb^{3+}}/c_{apoEoCen}$ is shown as the insert in Fig.1.

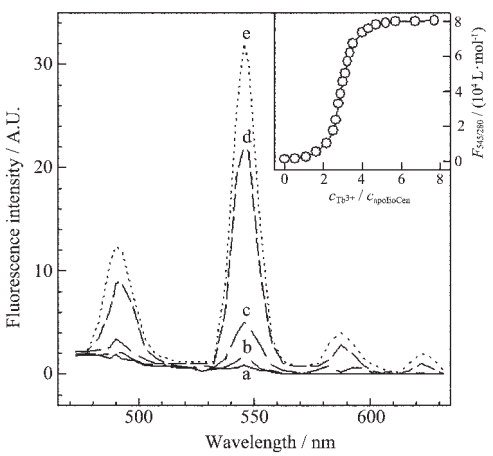

Fig.1 Tyrosine-sensitized fluorescence spectra for (a) free Tb^{3+} (6.2 μmol·L^{-1}) and for Tb^{3+} bound to apoEoCen at $c_{Tb^{3+}}$ / $c_{apoEoCen}$ of 1 (b), 2 (c), 3 (d), 4 (e), respectively

It can be seen that there are four-binding sites in apoEoCen for Tb^{3+}, the binding sites can be divided into two classes. At a stoichiometry of two Tb^{3+} ions per molecule of apoEoCen, there is little change in fluorescence, suggesting that Tb^{3+} binds first to the strong binding sites with weak sensitization for Tb^{3+} fluorescence. Addition of three and four ions of Tb^{3+} per molecule of apoEoCen produces large increases in fluorescence, two weak binding sites with strong sensitization for Tb^{3+} fluorescence. From an analysis of the amino acid sequence of apoEoCen, there is no tryptophan residue, the tyrosine (Tyr) 46 residue is located in the Ca^{2+}-binding site Ⅰ, and Tyr 79 residue in site Ⅱ, respectively, while the Ⅲ and Ⅳ are short of Tyr. The sensitization of Tb^{3+} fluorescence is originated from the energy-transfer from Tyr residues to the bound Tb^{3+}. So the binding sites Ⅲ and Ⅳ are the strong binding sites and the binding sites Ⅰ and Ⅱ are the weak binding sites. To investigate the cooperation between Ⅰ, Ⅱ binding sites and Ⅲ, Ⅳ binding sites, the *N*-apoEoCen with two weak binding sites was expressed and its binding properties were studied by Tb^{3+} fluorescence probe.

2.2 Binding of Tb^{3+} to *N*-apoEoCen

The fluorescence of Tb^{3+} was enhanced when the solution of *N*-apoEoCen was titrated by the addition of Tb^{3+} in 0.01 mol·L^{-1} Hepes, pH 7.4. Fig.2 shows the fluorescence titration curve of *N*-apoEoCen, the plot of F_M at 545 nm versus the total concentration of Tb^{3+}. Assuming that there are n Tb^{3+}-binding sites and they are independent and identical in *N*-apoEoCen, the conditional binding constant can be fitted using equa-

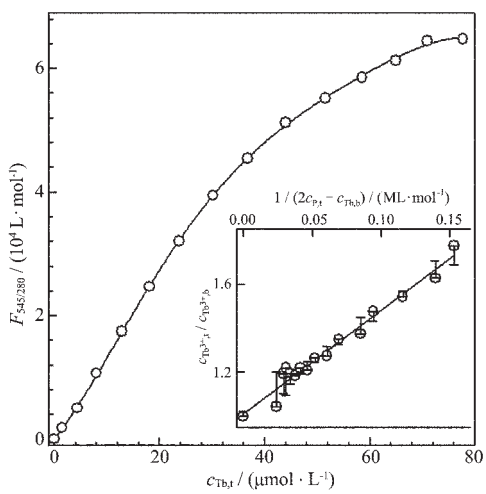

Fig.2 Titration curve for the addition of Tb^{3+} to *N*-apoEoCen in 0.01 mol·L^{-1} Hepes, pH 7.4. Insert: the plot of $c_{Tb^{3+},t}$ / $c_{Tb^{3+},b}$ versus $1/(2c_{N\text{-apoEoCen},t}-c_{Tb^{3+},b})$.

tion (**1**) from the data in Fig.2.

$$\frac{c_{\mathrm{Tb,t}}}{c_{\mathrm{Tb,b}}}=1+\frac{1}{K\{nc_{\mathrm{P,t}}-c_{\mathrm{Tb,b}}\}} \quad (\mathbf{1})$$

Where $c_{\mathrm{Tb,t}}$, $c_{\mathrm{Tb,b}}$ is the total, bound concentration of Tb^{3+}, respectively. $c_{\mathrm{P,t}}$ is the total concentration of protein[12]. In 0.01 mol·L^{-1} Hepes, pH 7.4, K (Tb_2-*N*-EoCen) can be calculated to be $(2.13 \pm 0.10) \times 10^5$ L·mol^{-1} and the numbers of binding sites are 2 for *N*-apoEoCen from the insert in Fig.2.

2.3 Binding of Ca^{2+} to *N*-apoEoCen

Ca^{2+} can make the fluorescence at 545 nm of Tb_2-*N*-EoCen quenched. Fig.3 is the fluorescence-quenching curve with the addition of Ca^{2+} to the solution of Tb_2-*N*-EoCen in 0.01 mol·L^{-1} Hepes, pH 7.4. It can be seen that Ca^{2+} in excess of 200-fold protein, 3 mmol·L^{-1}, makes the fluorescence at 545 nm quenched efficiently to 42%. Assuming that the fluorescence quenching comes from the competition of binding sites in *N*-apoEoCen between Ca^{2+} and Tb^{3+}, or the transformation from Tb_2-*N*-EoCen to Ca_2-*N*-EoCen, the relative binding constant of Ca_2-*N*-EoCen can be fitted from the fluorescence quenching curve in Fig.3 using equation **2**. The relative binding constant of Ca_2-*N*-EoCen, $K(Ca_2\text{-}N\text{-EoCen})$ is calculated to be $(7.52 \pm 0.02) \times 10^2$ L·mol^{-1} from the insert in Fig.3, in which c_{Ca} is replaced by $c_{\mathrm{Ca,t}}$.

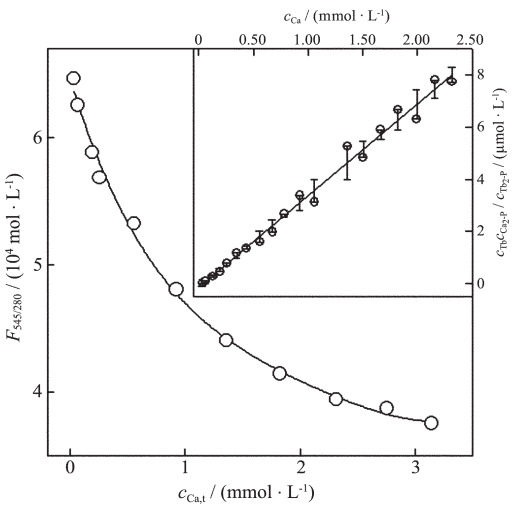

Fig.3 Fluorescence-quenching curve for the addition of Ca^{2+} to Tb_2-*N*-EoCen in 0.01 mol·L^{-1} Hepes, pH 7.4. The concentration of Tb_2-*N*-EoCen is about 18 μmol·L^{-1}. Insert is the plot of $c_{Tb^{3+}}c_{Ca^{2+}\text{-}N\text{-EoCen}}$ / $c_{Tb^{3+}\text{-}N\text{-EoCen}}$ versus $c_{Ca^{2+},t}$.

$$\frac{c_{\mathrm{Tb}}c_{\mathrm{Ca_2-P}}}{c_{\mathrm{Tb_2-P}}}=\frac{K_{(\mathrm{Ca_2-P})}}{K_{(\mathrm{Tb_2-P})}}c_{\mathrm{Ca}} \quad (\mathbf{2})$$

3 Conclusion

apoEoCen can bind two Tb^{3+} with high affinity and two Tb^{3+} with low affinity. The reactions between *N*-apoEoCen and Tb^{3+} or Ca^{2+} were studied by Tb^{3+} fluorescence probe and ionic competition in 0.01 mol·L^{-1} Hepes, at pH 7.4. On the basis of fluorescence titration curves, the conditional constant of Tb_2-*N*-EoCen was determined to be $(2.13 \pm 0.10) \times 10^5$ mol^{-1}·L and the relative constant of Ca_2-*N*-EoCen was calculated to be $(7.52 \pm 0.02) \times 10^2$ L·mol^{-1}.

References:

[1] JIANG Hui-Jun(姜慧君), WANG Xiao-Feng(王小锋), ZHOU Qing(周 青), et al. *J. Rare Earths*, **2002**,**20**(5):547~552

[2] Hu J, Jia X, Li Q, et al. *Biolchemistry*, **2004**,**43**:2688~2698

[3] Du X L, Zhang T L, Yuan L, et al. *Eur. J. Biochem.*, **2002**, **269**:6082~6090

[4] Zhao C G, Yang B S, Zhang W, et al. *Chinese Science Bulletin*, **2003**,**48**(2):127~130

[5] Schiebel E, Bornens M. *Trends Cell Biol.*, **1995**,**5**:197~201

[6] Hu H T, Sheehan J H, Chazin W J. *J. Biol. Chem.*, **2004**,**279** (49):50895~50903

[7] Ortiz M, Sanoguet Z, Hu H, et al. *Biolchemistry*, **2005**,**44**: 2409~2418

[8] Hu J, Yang X D, Wang K. *Chinese Chem. Lett.*, **2004**,**15**(10): 1197~1200

[9] Richardson F. *Chem. Rev.*, **1982**,**82**:541~552

[10]YANG Bin-Sheng(杨斌盛), BAI Hai-Jing(白海静), LIU Wen (刘 文), et al. *Huaxue Xuebao*(*Acta Chimica Sinica.*), **2002**, **60**(4):737~743

[11]HE Xiao-Jing(贺晓静), FENG Jiu-Ying(丰九英), WANG Wei(王 伟), et al. *Dongwu Xuebao*(*Acta Zoologica Sinica.*), **2004**,**50**(3):447~451

[12]Yang B S, Yang P, Song L H. *Chinese Science Bulletin*, **1984**, **29**(11):1502~1505