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Spectrochimica Acta Part A 67 (2007) 1189–1193
SPECTROCHIMICA ACTA PART A
www.elsevier.com/locate/saa

The spectral studies on the effect of Glu 101 to the metal binding characteristic of *Euplotes octocarinatus* centrin

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Received 4 July 2006; received in revised form 1 October 2006; accepted 5 October 2006

Abstract

Glu is highly conserved as the first amino acid of E-helix of the EF-hand protein. In this paper, Glu 101, the first amino acid of E-helix of the third EF-hand motif in *Euplotes octocarinatus* centrin (EoCen) was mutated to be Lys by the method of site direct mutation. Tb^{3+} and TNS were used as fluorescence probes in the study of the effect of this mutation to the metal binding characteristic of EoCen by fluorescence spectra. Results indicate that compared with EoCen, the mutation protein (E101K) displays a different Tb^{3+} binding characteristic and an increased hydrophobic exposure surface. Polyacrylamide gels electrophoresis indicated that the electrophoretic mobilities of EoCen and E101K are distinctly different. It can be deduced that the conformation of EoCen has been altered by this mutation. The general conditional binding constant of Tb^{3+} to the three loops of EF-hand sites I–III in E101K was calculated to be $(5.64 \pm 0.57) \times 10^5\ M^{-1}$ according to the modified equation of the single binding process.

Keywords: Centrin; Tb^{3+}; Fluorescence spectra

1. Introduction

The microtubule-organizing center (MTOC) is cytoplasmic organelles, encountered in almost all the eukaryotic cells and having an important role in the nucleation of the microtubules and the regulation of their dynamics [1]. In higher eukaryotes, such as humans, the centrosome functions as the MTOC. Lower organisms possess equivalent MTOCs too, notably the basal body in algae and the spindle pole body (SPB) in yeast. The composition of MTOCs is highly proteinaceous, and there exists significant structural heterogeneity among them in different eukaryotes. Despite this heterogeneity, MTOCs in all eukaryotes contain a number of conserved protein components. Among them, two proteins are of particular interest since both molecular and functional data are available. One of the proteins, γ-tubulin, is thought to be a universal marker for MTOCs. The other protein, centrin, is a 20 kDa acidic protein that belongs to the calcium-binding EF-hand superfamily (also named as calmodulin superfamily) [2–4]. Like calmodulin (CaM), centrin has two similar globular domains, the N-terminal and the C-terminal domains, linked by a central helix. Each domain contains two Ca^{2+}-binding sites (EF-hand) with a 12-residue loop (EF-loop) flanked by two α-helices known as the E and F helices [5]. Whereas centrin has a very limited number of known cellular targets relative to CaM, implying that it may have a more specialized biological function in the cell [6]. Centrin is first identified as a major component of the fibers that link the nucleus to the flagellar apparatus in flagellated unicells [7]. Centrin seems to act as a Ca^{2+} sensor, i.e., in its Ca^{2+}-load form, centrin interacts with specific target protein to modulate the cellular activity [8]. In general, the binding of Ca^{2+} involves a structural rearrangement of the α-helices of the EF-hand pair domain with the consequent exposure of a hydrophobic cleft [9].

Ln^{3+} ions have ionic radii (Tb^{3+} 0.923 Å and La^{3+} 1.03 Å) and coordination properties similar to Ca^{2+} (0.99 Å) [10]. On the other hand, Tb^{3+} can accept the energy from the excited donor, such as the aromatic ring of Trp or Tyr in proximity and its fluorescence enhancement at 545 nm can be observed [11]. Hence, Tb^{3+} effectively substitutes for Ca^{2+} and has been used to investigate the intrinsic metal-binding properties of individual sites in CaM [12–16].

Abbreviations: *C. reinhardtii*, *Chlamydomonas reinhardtii*; EoCen, *Euplotes octocarinatus* centrin; E101K, the mutant protein at 101 position; HsCen 2, human centrin 2

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doi:10.1016/j.saa.2006.10.006

Table 1
The amino acids sequence of E101K. ***K*** is the mutative Lys at position 101, E is the Glu at position 100 and Y is Tyr at position 46, 72 or 79

MIKKPEFGLMQPP
E-helix Ca^{2+}-loop F-helix
28.....EF-hand I 56
KKRVRQELSEEQKQ EIKEAFDLF **DTNKTGSIDYHE** LKVAMRAL
64.....EF-hand II 92
GFDVKKP EILELMNEY **DREGNGYIGFDD** FLDIMTEK
101.....EF-hand III.....129
IKNRDPVE***K***ILKAFKVF **DEDNSGKISLRN** LKRVAKEL
137..... EF-hand IV.....165
GENLSDD ELQAMIDEF **DKDQDGEISEQE** FLNIMKQTSIY

The canonical sequence for the E-helix, based on the known amino acid sequences of more than 150 members of the CaM superfamily, can be represented as En**nn**n-with E being glutamate, n a hydrophobic amino acid, and * any amino acid [17,18]. Thus, the Glu at the beginning of the E-helix would be expected to be important to the proper conformation and function of centrin. Taillon et al. [19] have reported that Glu 101 in the *C. reinhardtii* centrin was replaced by some different amino acids through the method of UV mutagenesis, then the location of this protein were differently affected. However, to my knowledge, the exhaustively study of the effect of Glu 101, the first amino acid of E-helix of site III in EoCen, to the metal binding characteristics of this protein by physicochemical methods has not been reported.

EoCen is the first reported by our laboratory (gene register Y18899) [20]. In this paper, the mutant protein (E101K) with Glu 101 changing to be Lys (Table 1) was obtained by the method of site direct mutation. Results indicate that the metal binding characteristic of EoCen has been altered largely by this mutation. The average conditional binding constant of Tb^{3+} binding to the loops of sites I–III was calculated to be $(5.64 \pm 0.57) \times 10^5\ M^{-1}$.

2. Materials and methods

2.1. Materials

N-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) (analytical reagent), ampicillin (ultra pure grade), isopropyl-β-D-thiogalactoside (IPTG) (ultra pure grade), tryptone, and yeast extract were bought from Bio. Basic Inc. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) was bought from Sigma Chemical Co. and used without further purification. Glutathione Sepharose™ 4B (GST) was purchased from Pharmacia Ltd.

A pair of primers, p1 (5′-AAAGCATTCAAGGTATTTGATGAA-3′) and p2 (5′-AAGAATTTTTTCTACAGGATCTCT-3′), were synthesized by Biological company of Shanghai (China).

Terbium oxide (99.99%) was obtained from Rare Earth Research Institute of Hunan (China). The stock solution of terbium (Tb^{3+}) was prepared in distilled water with a small amount of concentrated hydrochloric acid and its concentration was standardized by the titration of EDTA.

2.2. Protein engineering, expression and purification

The EoCen was obtained as previously reported [20]. The E101K was acquired by polymerase chain reaction technique with p1 (5′-AAAGCATTCAAGGTATTTGATGAA-3′) and p2 (5′-AAGAATTTTTTCTACAGGATCTCT-3′) used as primers. The resultant PCR product was subcloned into a PGEX-6P-1 expression vector. The sequence of the clone was confirmed by commerical company. The mutant clone was transformed into BL21(DE3) cells *Escherichia coli* strains induced with IPTG to yield milligram quantities of the desired protein. Transformation and expression were performed with modifications to the protocol of Baron [21]. The aimed proteins were identified via SDS-PAGE and were further purified by HPLC.

2.3. Metal ion removal and protein concentration

The EoCen and E101K were precipitated with 3% trichloroacetic acide and passed through a 40 cm × 1 cm Sephadex G-75 column equilibrated in 50 mM Tris–HCl (pH 7.5) and 150 mM KCl. The protein samples were concentrated through ultrafiltrated extensively against 10 mM Hepes, pH 7.4 and 4 °C, and the concentration of the stock solutions were measured by the spectrophotometrically absorption at 280 nm with a molar extinction coefficient of $\varepsilon_{280} = 5600\ M^{-1}\ cm^{-1}$ which was estimated from the Tyr and Trp contents as previously reported [22].

2.4. Aromatic residue-sensitized Tb^{3+} energy transfer

The protein solutions (6.0 μM) were prepared by dilution of the stock solution with 10 mM Hepes pH 7.4 and 150 mM KCl. Transferring 2.0 mL above solution into a 1 cm quartz cell, adding Tb^{3+}stock solutions directly to it. The mixture were shaken thoroughly, and then equilibrated for 3.0 min at 25 °C in order to make the binding of Tb^{3+} to protein was complete. The aromatic residue-sensitized Tb^{3+} energy transfer fluorescence spectra were measured by HITACHI 850 fluorescence spectrophotometer. A glass filter with a long pass of >430 nm was used to avoid secondary Raleigh scattering. The slit width of excitation and emission were both set at 10 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 EoCen.

2.5. Interaction with TNS in the present of 0–4 equiv. of Tb^{3+}

The metal-dependent changes of the hydrophobic exposure degree of EoCen and E101K were studied by monitoring the fluorescence emission spectra of the hydrophobic probe TNS. The fluorescence spectra of TNS were measured by HITACHI

F-2500. The excitation wavelength was set at 320 nm. To correct the dilution, the fluorescence intensity was converted to molar fluorescence intensity by dividing the fluorescence intensity via the analytical concentration of EoCen. The protein solutions (6.0 μM) were prepared by dilution of the stock solution with 10 mM Hepes pH 7.4 and 150 mM KCl. Add different equivalents Tb^{3+} to protein solutions in advance, five minutes later, transferring 2.0 mL above solution into a 1cm quartz cell. The TNS stock solution was gradually added to the mixed solution. The mixture were shaken thoroughly, and then equilibrated for 3.0 min at 25 °C in order to make the binding of TNS to protein was complete before measurements were taken.

2.6. Gel electrophoresis

The electrophoretic mobility of protein was evaluated by nondenaturing polyacrylamide gel electrophoresis under discontinuous conditions as a modified technique described by Beckingham [23]. Polyacrylamide gels contained 373 mM Tris (pH 8.8), 1% ammonium persulfate, and 16% acrylamide/bis (29:1). To induce polymerization, 0.05% TEMED was added. Tris–glycine electrophoresis running buffer contained 25 mM Tris (pH 8.3), 250 mM glycin. All electrophoresis was at room temperature. Gels were run at a constant current of 11–12 mA for 2 h.

3. Results

3.1. The binding of Tb^{3+} to EoCen and E101K

Fig. 1 shows the titration curves of the addition of Tb^{3+} to EoCen (curve 1) and E101K (curve 2), respectively. As displayed in curve 1, the first two equivalents of added Tb^{3+} appear to bind quantitatively, but with minimal sensitization. As additional two equivalents of Tb^{3+} were added, considerable sensitized emission is observed. It indicates that the weak fluorescence sensitivities were obtained when the first two equivalents Tb^{3+} were added to EoCen, whereas the stronger fluorescence sensitivities were induced with the addition of another two equivalents Tb^{3+}. In curve 2, the height of the beginning segment of this curve was similar to the corresponding part of curve 1. However, curve 2 appears a visible rising after $r(r = [Tb^{3+}]/[\text{protein}]) = 1$ and gets to a higher position which is little lower than that of curve 1 after $r = 4$. It illuminates the fluorescence sensitivity of Tb^{3+} binding to the second binding site of EoCen and E101K are clearly different.

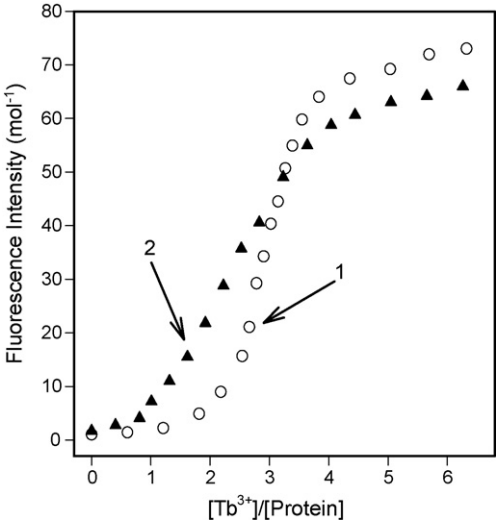

Fig. 1. The titration curves of the addition of Tb^{3+} to 6.0 μM EoCen (curve 1) and 6.0 μM E101K (curve 2) by measuring the fluorescence intensity at 545 nm under the conditions of 10 mM Hepes pH 7.4, 150 mM KCl and 25 °C.

3.2. Interaction with TNS in the present of 0–4 equiv. of Tb^{3+}

Equimolar hydrophobic probe TNS were gradually added to EoCen and E101K sample solutions, which were in the presence of different (from 0 to 4) equivalents Tb^{3+}, then the TNS fluorescence intensity at 430 nm were mesured (Fig. 2a and b). In Fig. 2a, curves 1 and 2 were under curve 3 and the three curves

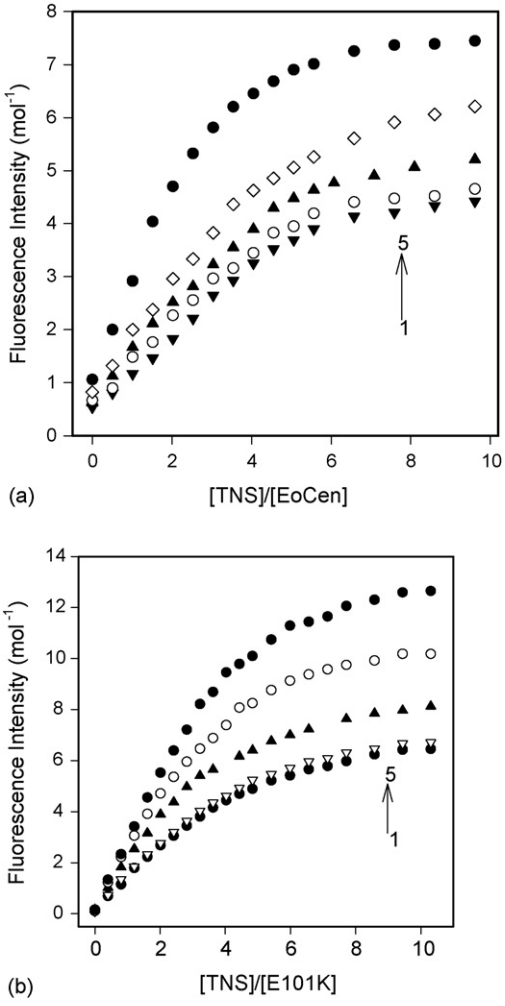

Fig. 2. The titration curves of the addition of TNS to 6.0 μM EoCen (figure a) and 6.0 μM E101K (figure b) in the presence of 0, 6.0, 12.0, 18.0, and 24.0 μM Tb^{3+}, respectively (in figure a: curves 3, 1, 2, 4, 5 and in figure b: from curves 1 to 5), by measuring the fluorescence intensity at 430 nm under the conditions of 10 mM Hepes pH 7.4, 150 mM KCl and 25 °C.

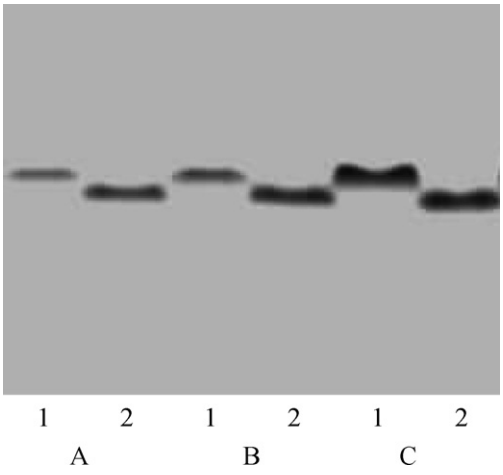

Fig. 3. Nondenaturing gel electrophoresis of EoCen and E101K under the conditions of 10 mM Hepes pH 7.4. (A) Relative mobilities of 2.0 nM E101K (lane 1) and 3.0 nM EoCen (lane 2). (B) Relative mobilities of 4.0 nM E101K (lane 1) and 6.0 nM EoCen (lane 2). (C) Relative mobilities of 8.0 nM E101K (lane 1) and 9.0 nM EoCen (lane 2).

were almost at the same height, but the positions of curves 4 and 5 were ordinal raised and were both distinctly higher than curve 3. In Fig. 2b, only curves 1 and 2 were near to each other. The other three curves were all ordinal increased distinctly. On the other hand, the fluorescence intensity which were showed by curve 3 in Fig. 2a was obviously lower than that of curve 1 in Fig. 2b.

3.3. Nondenaturing polyacrylamide gel electrophoresis

Fig. 3 shows the results of nondenaturing polyacrylamide gel electrophoresis of E101K and EoCen at different concentrations. It can be seen that the electrophoretic mobility of E101K migrate were all detectably slower than EoCen in each group.

3.4. The calculation of the general conditional binding constant

As displayed in Fig. 1, the slope of curve 2 within the range of $1 < r < 4$ can be regarded as unaltered. In other words, the Tb^{3+} dependence of the fluorescence intensity changes is very similar when Tb^{3+} binding to the loops of the EF-hand sites I–III in E101K. It was suggested that the conformational changing were induced simultaneously by the same binding event. The K_d value of Tb^{3+} bind to the three loops of the protein was calculated by fitting the metal titration curves, assuming that the changes are from both specific and non-specific binding, using the following equation modified from that of single binding process [24]:

$$\Delta S = \Delta S_{\max} \frac{([P]_T + [M]_T + K_d) - \sqrt{([P]_T + [M]_T + K_d)^2 - 4[P]_T[M]_T}}{2[P]_T} + C[M]_T \quad (1)$$

where ΔS and $\Delta S_{\max}$ are the signal change and the total signal change from the specific binding, respectively; C the contribution from the non-specific binding; $[M]_T$ and $[P]_T$ are the total concentrations of metal ion and protein, respectively. The effects from the non-specific binding are simplified to be linear at the experimental metal concentration since this binding is very weak.

The general conditional binding constant was calculated as $(5.64 \pm 0.57) \times 10^5\ M^{-1}$, which was about two folds higher than the Tb^{3+} binding constant of N-terminal of EoCen [25].

4. Discussions

According to the amino acids sequence, there are two aromatic residues, Tyr 46 and Tyr 79, in the N-terminal loops of EoCen, while there is no any aromatic residue in the C-terminal loops. Based on the aromatic residue-sensitized non-radialization energy transfer theory[11], it can be deduced that binding Tb^{3+} to C-terminal domain of EoCen would induce the weaker fluorescence sensitivity than binding to N-terminal domain. As can be seen in Fig. 1, curve 1 indicates that Tb^{3+} binds to C-terminal domain of EoCen are preferable than N-terminal domain. Using the *C. reinhardtii* centrin and HsCen 2 as target molecules, respectively, the same conclusion had been obtained by Chazin and coworkers [26] and Craescu and coworkers [27]. Since the site IV in *C. reinhardtii* centrin bound Ca^{2+} was about 100-fold more strongly than site III [28], it could be conjectured that the first equivalent Tb^{3+} was binding to the site IV of EoCen and the second equivalent Tb^{3+} was binding to site III. As the mutated Glu is the first amino acid of E-helix of site III in EoCen and compared with curve 1 in Fig. 1, curve 2 appears a visible rising after $r = 1$, we assurance two of the following conclusions: (1) the second equivalent Tb^{3+} was binding to site III; (2) the metal-binding characteristic of EoCen has been altered by the mutation of Glu to Lys at position 101.

TNS was used as a hydrophobic probe in the following experiment. Fig. 2a indicates that a decreased hydrophobic exposure degree was induced by one or two equivalent Tb^{3+}-saturated EoCen and the conformation change of the protein which were induced by the binding with Tb^{3+} was slight. When the additional Tb^{3+} was added, the hydrophobic exposure surface became larger and the conformation changed obviously. Compared with Fig. 2a with b shows that the binding of the second equivalents Tb^{3+} to E101K has induced more exposured hydrophobic surface and a larger conformation change. This result also approved the two conclusions above.

In Fig. 2, the fluorescence sensitivity that was induced by the binding of equimolar TNS to E101K (curve 1 in Fig. 2b) is obviously stronger than binding to EoCen (curve 3 in Fig. 2a). That is to say, the mutation protein displays larger hydrophobic exposure surface than EoCen. Moreover, the results of the native gel electrophoresis have shown that there is detectably difference in the mobility of EoCen and E101K. Combined with the comparison of the general conditional binding constant of Tb^{3+} binding to sites I–III in E101K and the Tb^{3+} binding constant of N-terminal of EoCen [25], We deduced that the conformation of the EoCen has been changed by this mutation of Glu to be Lys at position 101.

Since little of the structure of EoCen at atomic level was known, how this result was induced would not to be told with certainty. However, according to the highly conserved nature of Glu 101 and the X-ray diffraction analysis of a similar protein [29], it can be speculated that Glu 101 in the EoCen forms a salt bridge with Arg 96, which stabilizes the structure of the centrin. But the dramatic change in charge caused by the substitution of Lys at position 101 in E101K would make it impossible to form this salt bridge. Maybe the Glu 100 or/and other potential amide acids would make an intensively interaction with Lys 101 and cause the protein to assume a different conformation. Then, the energy transferring from Tyr 79 or/and the Tyr 72 (Table 1) to Tb^{3+}, which were bound to site III in E101K would be induced by this changed conformation.

5. Conclusion

The experiments of Tb^{3+} fluorescence sensitivity and the binding of proteins to hydrophobic probe TNS were performed in this paper. The results indicate E101K displays a different Tb^{3+} binding characteristic and an increased hydrophobic exposure surface. Combined with the result of polyacrylamide gel electrophoresis and the general conditional binding constant of Tb^{3+} binding to the three loops of EF-hand sites I–III in E101K, we deduce that the conformation of EoCen has been affected by the mutation of Glu to Lys at the position 101.

Acknowledgements

This work was supported by the National Natural Science Foundation of PR China (No. 20371031) and the Natural Science Foundation of Shanxi Province (No. 20031017).

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