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Investigation on the binding of TNS to centrin, an EF-hand protein

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Abstract

The interaction between 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) and *ciliate Euplotes Octocarinatus* centrin (Cen) has been studied by fluorescence spectroscopy. The binding constants of TNS with Cen were measured at different temperature in the 0.01 M Hepes, pH 7.4. The binding process is exothermic and involves a positive entropy change. The negative value of enthalpy predominately contributes to the negative free energy of binding between TNS and Cen. The salt (KCl) increases the association constant of TNS and Cen. These results and resonance light scattering experiment suggest that the binding force between TNS and Cen is hydrophobic. The distance (r) between TNS and tryptophan of mutant G115W, which sheds more insight into the binding of TNS to Cen, was determined as 4.85 nm based on Förster non-radiative energy transfer theory.

Keywords: TNS; Cen; Energy transfer

1. Introduction

It is well known that the quantum yield of fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) and related compounds, such as 1-anilino-8-naphthalenesulfonate (ANS), is very much higher in a nonpolar than in a polar solvent. Also, the emission spectrum in a nonpolar solvent is blue shifted relative to the spectrum in polar solvents [1,2]. This characteristic is, therefore, widely used to detect protein conformational change assuming that the environments of dye binding sites change as protein conformation changes.

Centrins, also known as caltractin, are a closely related subfamily within the larger superfamily of Ca^{2+}-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^{2+} [3], as shown in Fig. 1 [4]. Upon Ca^{2+}/Tb^{3+} binding, centrin undergo a large conformational change and concomitant hydrophobic surface exposure. TNS has been extensively used in the conformational change of centrin induced by metal ions [5–8]. Cen has also the similar property and can increase the fluorecence intensity of TNS when binding to TNS. Cen is firstly reported by our laboratory [9] (gene register Y18899), which is cloned from *Euplotes Octocarinatu* and contains four tyrosine residues but no tryptophan residue. The mutant G115W (the glycine in position 115 located in wild type protein's third EF-hand was converted into tryptophan) was obtained to calculate the distance of TNS and third EF-hand of Cen using the energy transfer from tryptophan to TNS, because energy transfer between tryptophan and TNS is much easier.

Though TNS has been extensively used in the study of centrin, the actual probe-binding environment is still obscure, and the nature of the binding forces is not well defined. In this report, we determined the acting force of TNS and Cen and energy transfer between TNS and G115W in 0.01 M Hepes, pH 7.4. The results showed the acting force of TNS and Cen mainly belongs to hydrophobic interaction and distance of TNS and tryptophan in G115W is 4.85 nm, which can provide more evidences to understand the nature of the binding force of TNS

Abbreviations: Cen, *ciliate Euplotes Octocarinatus* centrin; G115W, the mutant protein at 115 position of Cen from glycine to tryptophan; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; RLS, resonance light scattering.

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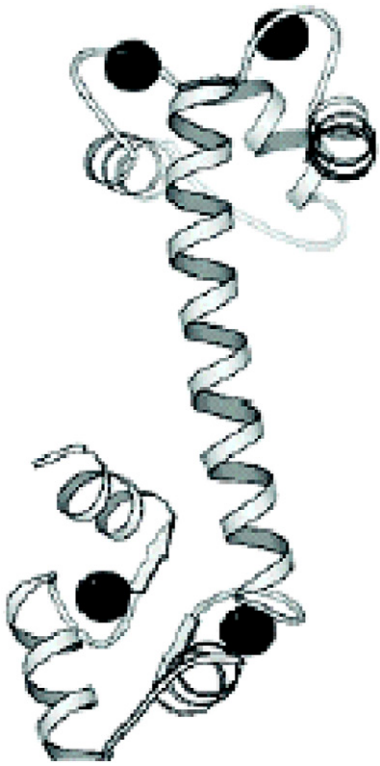

Fig. 1. Three-dimensional structure of centrin represented by calmodulin [2]. Ca^{2+} ions are shown as dark spheres (a three-dimensional structure of intact centrin is not yet available).

and EF-hand protein and may help to research fluorescence probe.

2. Materials and methods

2.1. Reagents

Hepes buffer, salts, and other chemicals utilized in protein purification were reagent grade. The purity of Tb_4O_7 is more than 99.9%, TNS was purchased from Sigma.

2.2. Stock solutions

A stock solution of terbium was prepared by dissolving weighed Tb_4O_7 in concentrated hydrochloric acid, which was standardized by complexometric titration with EDTA using xylenol orange as indicator in HAc/NaAc buffer at pH 5.5. The solution of TNS prepared by dissolving weighed samples.

2.3. Protein expression and purification

Cen was expressed and purified as described previously [9]. Using the full-length gene of Cen as template, the G115W mutant clone was obtained by PCR, and primers are 5′-ctgagaaatttaaagagagtt-3′ and 5′-gcttattttccttgaattatc-3′. 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 for expression and purification in the simliar way to the wide type Cen. The protein concentrations were measured spectrophotometrically using molar extinction coefficients at 280 nm of 5600 M^{-1} cm^{-1} for Cen and 11490 M^{-1} cm^{-1} for the mutant G115W. The extinction coefficient of protein was estimated from the tyrosine (Tyr) and Trp content as described by Pace et al. [10].

2.4. Fluorescence spectroscopy

All fluorescence experiments were performed on a Hitachi F-2500. To avoid contamination from tyrosine emission in the energy transfer experiment, protein samples were excited at 295 nm, a wavelength at which the absorption of this residue is negligible. For titration process in the presence of TNS, 320 nm was selected to be excitation wavelength.

2.5. Resonance light scattering measurements

Solution turbidity changes induced by the addition of metal ions were monitored by scanning synchronously with the same excitation and emission wavelength in the wavelength range of 310–500 nm.

3. Results and discussion

3.1. Characterization of the fluorescence in TNS–protein complex and the binding constants

As shown in Fig. 2, TNS had a weak fluorescence in water alone, and the fluorescence intensity increased largely with binding to Cen, accompanied by a blue shift of the maximum peak from 500 to 440 nm. The titration curves of TNS to Cen at different temperature had been obtained, possibly indicating the formation of the TNS–protein complex, can be seen in Fig. 3.

Given that there are n TNS-binding sites and they are independent and identical in TNS–protein complex [11]. The binding equation is presented by:

$$\mathrm{P} + n\mathrm{TNS} \leftrightarrow \mathrm{TNS}_n\mathrm{P} \tag{1.1}$$

Assuming the F_∞ is maximum molar fluorescence intensity, F_r is fluorescence intensity of every titration dot. The increase of fluorescence intensity resulted from the binding of TNS to protein. The following equations can be obtained:

$$F_\infty \propto n[\mathrm{P}]_t \quad F_r \propto [\mathrm{TNS}]_b \tag{1.2}$$

$$[\mathrm{P}]_b = \frac{F_r}{F_\infty}[\mathrm{P}]_t \tag{1.3}$$

$$[\mathrm{TNS}]_b = n[\mathrm{P}]_b \tag{1.4}$$

where $[\mathrm{TNS}]_b$ is the bound concentration of TNS. $[\mathrm{P}]_t$ and $[\mathrm{P}]_b$ is the total and bound concentration of protein, respectively. The binding constant can be given as follows:

$$K = \frac{[\mathrm{TNS}]_b}{[\mathrm{TNS}]_f n[\mathrm{P}]_f} \tag{1.5}$$

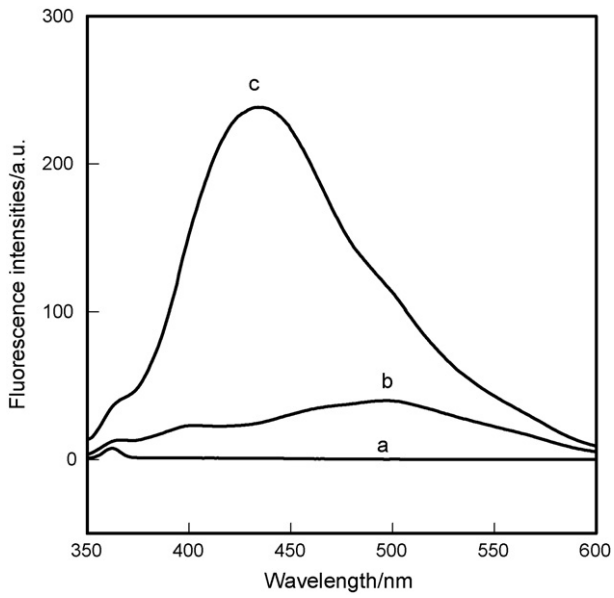

Fig. 2. The fluorescence of TNS in the absence (b) and in the presence (c) of Cen. 'a' is blank.

$[TNS]_f$ and $[P]_f$ represent the free concentration of TNS and protein, respectively. Finally, equation can be expressed by (1.6)

$$\frac{[TNS]_t}{[TNS]_b} = 1 + \frac{1}{K\{n[P]_t - [TNS]_b\}} \quad (1.6)$$

$1/K$ can be obtained by the linear slope of the plot of $[TNS]_t/[TNS]_b$ versus $1/n[P]_t - [TNS]_b$, as shown in Fig. 4. n was set to be 2, the intercept of Eq. (1.6) is very close to 1, and can also be seen in Fig. 3, $n = 2$ is suitable. The binding constants of TNS and Cen at different temperature were listed in Table 1. From which, the conclusion can be drawn that the binding constant decrease with the increase of temperature,

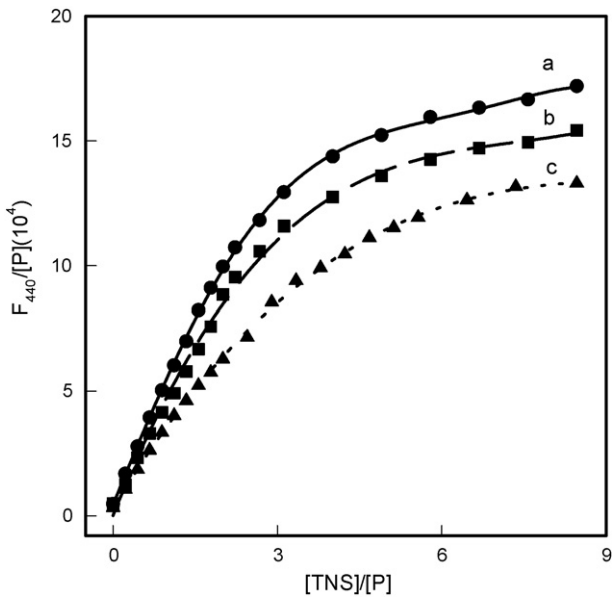

Fig. 3. The titration curve of Cen with the addition of TNS at different temperature, in 0.01 M Hepes, pH 7.4. a, b, c, represent 297 K, 303 K, 308 K, respectively. [Cen] = 9.100 μM, [TNS] = 253.4 μM.

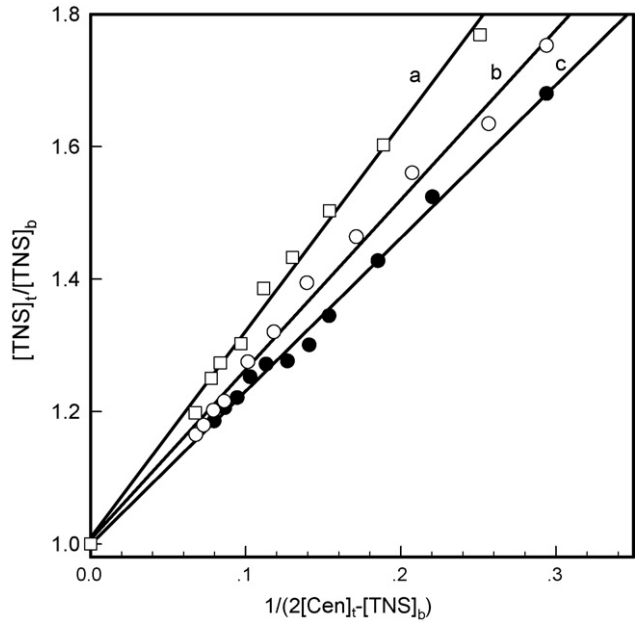

Fig. 4. The plot of ($[TNS]_t/[TNS]_b$) versus ($1/(2[Cen]_t - [TNS]_b)$). (a) 308 K; (b) 303 K and (c) 297 K.

indicating that the reaction is exothermic, higher temperature is harmful.

3.2. The determination of the force acting between TNS and Cen

TNS has been extensively used as hydrophobic probe in many studies. However, other researchers had reported some different results about the binding mode, owing to the sulfonate SO_3^- group, electrostatic interaction is most interaction in some cases. So we carried out the following experiment to solve this problem.

3.2.1. The determination of the force acting between TNS and Cen

The acting forces between a small molecule and a biomolecule belong to the weak interaction of molecules including hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force and so on [12]. Because the temperature changes were very little, the enthalpy change can be regarded as a constant. ΔG can be calculated from the following Eq. (2.3), further ΔH and ΔS can be obtained by Vant–Hoff Eq. (2.2).

$$\ln\frac{k_2}{k_1} = \frac{(T_2 - T_1)}{T_1 T_2}\frac{\Delta H}{R} \quad (2.1)$$

Table 1
The binding constants and thermodynamic parameters of between TNS and Cen at different temperature

T/K	K (10^5 mol^{-1} L)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol^{-1})
297	7.70 ± 0.50	−33.46 ± 0.16		
303	6.55 ± 0.28	−33.07 ± 0.14	−63.66 ± 7.17	101.5 ± 2.37
308	4.85 ± 0.12	−32.33 ± 0.06		

$$\Delta G = \Delta H - T\Delta S \quad (2.2)$$

$$\Delta G = -RT \ln K \quad (2.3)$$

where the values of ΔH, ΔG and ΔS are enthalpy change, free energy change and entropy change, respectively. Can be seen in Table 1, $\Delta G < 0$, $\Delta S > 0$ showed that the binding of TNS to the hydrophobic patch of Cen releases the water molecule located in hydrophobic patch before and this process is spontaneous and free energy change decrease. Entropy change $\Delta S > 0$, and enthalpy change $\Delta H < 0$, it can be deduced that the acting force for the binding reaction between TNS and Cen is mainly a hydrophobic interaction force based on the reported early [13,14]. The negative value of enthalpy predominately contributes to the negative free energy of binding between TNS and Cen. However, other research reported that if $\Delta S > 0$, the case can be divided into two kind modes. One is hydrophobic interaction; the other is electrostatic binding [15]. So the following experiment is necessary.

3.2.2. *The influence of the ionic strength on the binding of TNS to Cen*

The influence of the ionic strength on the fluorescence intensity of TNS in the presence of Cen had been studied, in which KCl was used to control the ionic strength. As shown in Fig. 5, the fluorescence intensity change of TNS induced by the different KCl concentration can be classified into two phases. First, the fluorescence intensity increased with the increase of the concentration of KCl in the range of 0–70 mM; second, decreased when KCl concentration is up to 70 mM. The salt (KCl) increased the association constant of TNS and Cen in the first phase, may be owing to increasing the positive entropy change, which resulted in the increase of TNS fluorescence intensity, which also proves our viewpoint that the hydrophobic force is the dominant form in the binding process of TNS to Cen. The high ionic strength was advantageous to the hydrophobic process but disadvantageous to the electrostatic binding [13]. In the second phase, the decrease intensity may be attributed to the following reasons: (1) much higher ion strength might change protein conformation which influenced the binding process; (2) the electrostatic forces might be the dominant factor. The hydrophobic force and electrostatic force competed with each other and could transit to each other when the experimental conditions changed. When KCl concentration was more than certain value (may be 70 mM), the Cl^- competed with TNS to bind the Cen through ionic interaction, which would reduce the number of TNS molecules binding to the Cen.

Using the similar method of Sections 3.1 and 3.2.1, the binding constants and the thermodynamic parameters of the interaction of TNS and Cen in the presence of 40 mM and 70 mM KCl, had been obtained, respectively. The results showed further that increase of ionic strength can improve the binding of TNS to Cen and hydrophobic interaction is dominant in the binding of TNS to Cen.

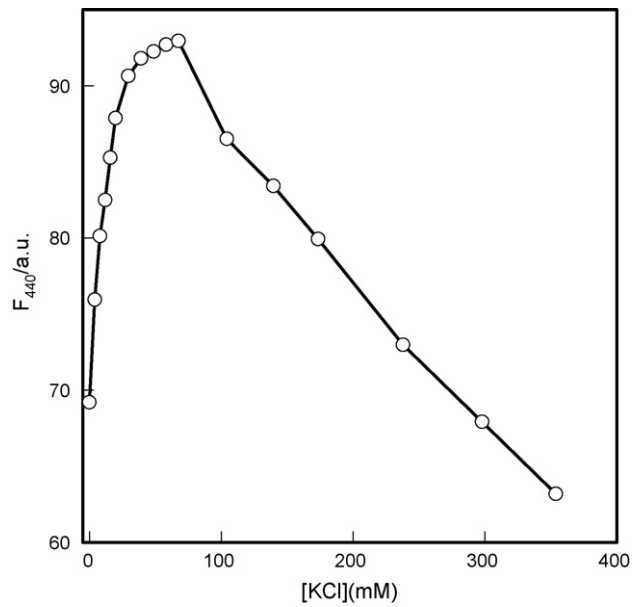

Fig. 5. The fluorescence intensity change of TNS–Cen complex influenced by the ionic strength at 0.01 M Hepes, pH 7.4. [Cen] = 11.30 μM, [TNS] = 25.34 μM.

3.2.3. *Further prove hydrophobic force mode using the resonance light scattering*

Centrins from yeast, algae *Scherffelia dubia*, or humans may form multimer/polymer in the presence of Ca^{2+} [16]. The hydrophobic surface area exposure of protein induced by the addition of metal ions plays an important role in the polmerization [17]. Can be seen in curve a of Fig. 6, after the addition of two equivalent Tb^{3+} (similar to Ca^{2+}), RLS intensity increased largely, which got to a plateau at about $[Tb^{3+}]/[Cen] = 4$. Curve b is similar to curve a, main difference being maximum value

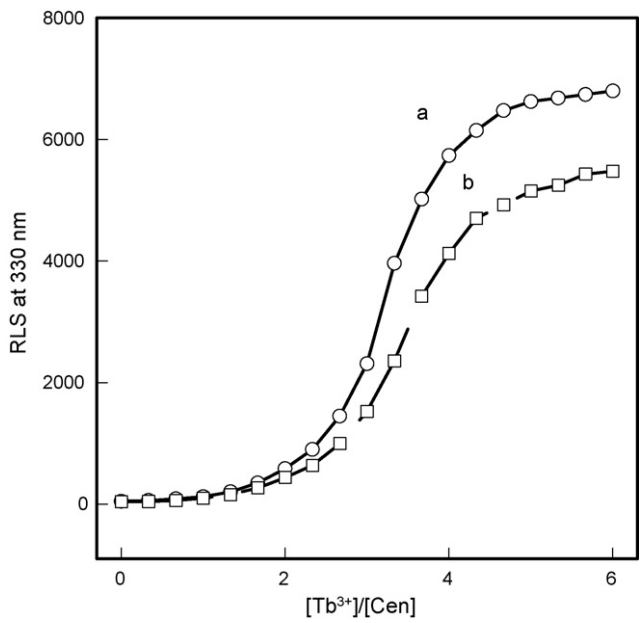

Fig. 6. The titration curve of (a) Cen and (b) TNS–Cen with the addition of $[Tb^{3+}] = 227.5$ μM using resonance light scattering value at 330 nm in 0.01 M Hepes, pH 7.4. [Cen] = 6.825 μM. [TNS] = 15.20 μM.

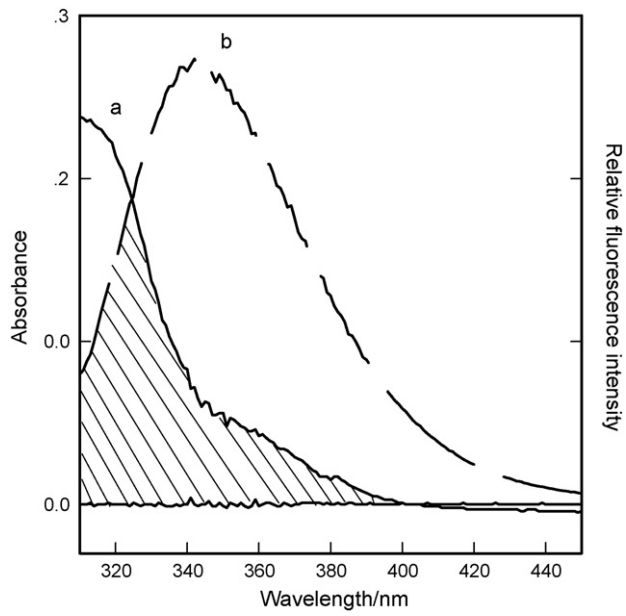

Fig. 7. Overlap of absorption spectra of TNS-G115W (a) and the fluorescence emission spectra of G115W (b) in 0.01 M Hepes, pH 7.4. Experimental conditions: G115W 8.700 μM and TNS 8.940 μM.

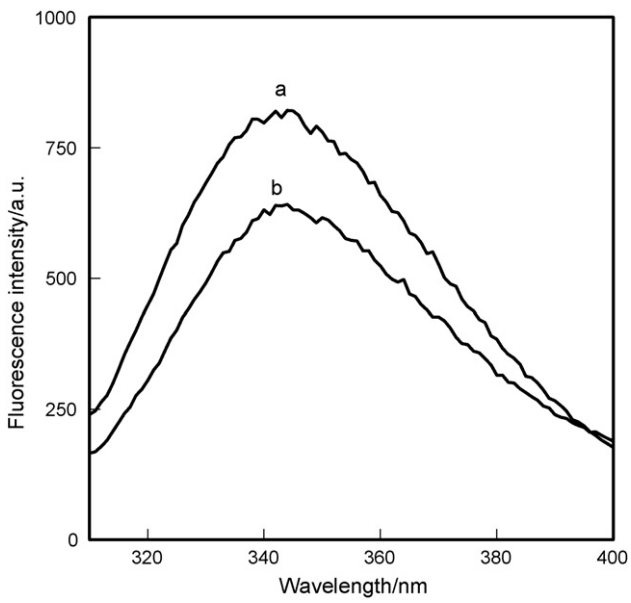

Fig. 8. The fluorescence spectra of G115W (a) in the absence and (b) in the presence of TNS in 0.01 M Hepes, pH 7.4. The concentration of G115W was 8.700 μM. TNS was 8.940 μM.

(RLS intensity), showing that the polmerization extent decreased in the presence of TNS. In other words, TNS occupied partly hydrophobic surface, leading to decrease the polymerization. From the other aspect, the conclusion can be drawn that binding of TNS and Cen dominated mainly by hydrophobic interaction.

3.3. Energy transfer between TNS and G115W

Förster energy transfer (FET), the transfer of energy from a donor to an acceptor via a dipole–dipole mechanism, has long been used to measure distances between a donor and an acceptor in proteins and other macromolecules [18]. According to the Förster non-radiative energy transfer theory [19,20], the energy transfer will happen under the following conditions: (1) the donor can produce fluorescence light; (2) fluorescence emission spectrum of the donor and UV absorbance spectrum of the acceptor have overlap; (3) the distance between the donor and the acceptor is approach and lower than 7 nm. G115W is a donor and it has a strong intrinsic fluorescence. It can be seen from Fig. 7 that energy transfer easily occurred between TNS and G115W for the large spectral overlap between the UV absorption spectra of TNS (a) and the fluorescence emission spectra of G115W (b). The energy transfer effect E is related not only to the distance between the acceptor and the donor, but also to the critical energy transfer distance, the relation between them is:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \tag{3.1}$$

where r is the distance between the acceptor and the donor, R_0 is the critical distance when the transfer efficiency is 50%, which can be calculated by:

$$R_0^6 = 8.79 \times 10^{-25} k^2 n^{-4} Q_D J(\lambda) \tag{3.2}$$

where k^2 is the spatial orientation factor of the dipole, n the refractive index of the medium, Q_D the fluorescence quantum yield of the donor, which can be calculated to be 0.18 using the absolute quantum yield (0.14) of Trp in aqueous solution [21] as standard value by the following equation:

$$\frac{Q_2}{Q_1} = \frac{F_2}{F_1}\frac{A_1}{A_2} \tag{3.3}$$

$J(\lambda)$ the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J(\lambda) = \frac{\int F(\lambda)\varepsilon_A(\lambda)\lambda^4 \Delta\lambda}{\int F(\lambda)\Delta\lambda} \tag{3.4}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, ε_A (λ) the molar extinction coefficient of the acceptor at wavelength λ. The energy transfer efficiency is given by:

$$E = 1 - \frac{F_{DA}}{F_D} \tag{3.5}$$

F_D, and F_{DA} represent relative fluorescence intensity of the donor (G115W) in the absence and presence of acceptor (TNS), respectively.

$J(\lambda)$ can be evaluated by integrating the spectra in Fig. 7 and Eq. (3.4) for $\lambda = 310$–400 nm and was 1.17×10^{-14} cm^3 M^{-1}. And the characteristic distance $R_0 = 3.96$ nm, using $k^2 = 2/3$, $n = 1.4$ [21]. The energy transfer effect $E = 0.23$ from Eq. (3.5) in Fig. 8, and the distance r between TNS and Trp residue in G115W was 4.85 nm.

The mutant G115W was built by replacing the glycine in position 115 by tryptophan, which is located in third EF-hand of C-terminal of Cen. Every EF-hand motif is Ca^{2+} binding site. Through determining the distance of TNS and tryptophan, more

details of TNS binding to EF-hand motif can be obtained, also provide the instruction to the use of TNS in the conformation change of Cen induced by Ca^{2+} or rare metal ions.

4. Conclusion

Using the fluorescence spectroscopy, the interaction of TNS and Cen has been studied. Through the calculated thermodynamic parameters of the interaction of TNS and the Cen, ion strength effect and resonance light scattering experiment indicating force acting between TNS and Cen is hydrophobic. According to the Förster non-radiative energy transfer, the distance r of TNS and tryptophan in G115W was calculated to be 4.85 nm. These experimental and theoretical data are of potential importance in understanding the mechanism of interaction between TNS and centrin and could be a useful guide for fluorescent probe research.

Acknowledgements

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