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Role of Asp37 in metal-binding and conformational change of ciliate *Euplotes octocarinatus* centrin

Wen Liu[1], Lian Duan[1], Bing Zhao[1], Ya-Qin Zhao[1], Ai-Hua Liang[2], Bin-Sheng Yang[1*]

[1]Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Shanxi University, Taiyuan, China; yangbs@sxu.edu.cn
[2]Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan, China

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ABSTRACT

Centrin is a member of the EF-hand super family that plays critical role in the centrosome duplication and separation. To investigate the role of Asp37 in the process of metal-binding and conformational change of ciliate *Euplotes octocarinatus* centrin (EoCen), the mutant D37K, in which aspartic acid 37 had been replaced by lysine, was first obtained by the site-directed mutagenesis. Then 2-p-toluidinylnaphthalene-6-sulfonate (TNS) was used as a fluorescence probe to detect the conformational change of the protein. The results show that the metal-binding capability of the site I of EoCen was lost by the mutation of Asp37→Lys. In comparison the Tb^{3+}-saturated EoCen, the hydrophobic surface of D37K, which is exposed by the binding of Tb^{3+}, has shrunk sharply, suggesting that Asp37 plays an important role in maintaining the proper conformation of EoCen in the presence of Tb^{3+}. Meanwhile, the conditional binding constants of TNS with Tb^{3+}-loaded EoCen and D37K were obtained, $K(Tb^{3+}\text{-EoCen-TNS})=(7.38\pm0.25)\times10^{5}\ M^{-1}$ and $K(Tb^{3+}\text{-D37K-TNS})=(1.16\pm0.05)\times10^{6}\ M^{-1}$.

Keywords: Centrin; Aspartic Acid; Tb^{3+}; TNS

1. INTRODUCTION

Centrin is a calcium-binding protein of ~20 kDa, and present in both the pericentriolar material and the centrioles of centrosome. Genetic studies show that centrin is essential to normal cell cycle-dependent duplication and segregation of the microtubule organizing center (MTOC) [1]. 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 [2]. It is fundamental to many cellular processes, including chromosomal segregation, cytokinesis, fertilization, cellular morphogenesis, cell motility, and intracellular trafficking [3]. Much research has focused on these functions, because abnormal centrosome duplication may lead to chromosomal instability and then cancer, an idea supported by discovery of supernumerary abnormal centrosomes in different human tumor cells [4-6]. In addition, centrin forms part of the human heterotrimetric DNA damage recognition complex required for global genome nucleotide excision repair [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. 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 [8,9]. Conformational changes are intrinsic to the function of a variety of proteins. 2-p-Toluidinylnaphthalene-6-sulfonate (TNS) has been extensively used in the conformational change of centrin induced by metal ions [10-12].

Ciliate *Euplotes octocarinatus* centrin (EoCen) 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 EF-hand protein calmodulin (CaM). 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 [13]. As show in **Figure 1** [14], the first amino acid of Ca^{2+}-binding 12-residue loop is aspartic acid which is highly conserved. Thus, the Asp at the first position of the loop would be expected to be important to the proper conformation and metal binding characteristics of centrin.

Lanthanides have been known for their diversity in biological effects, and the application of lanthanides in medicine has high potential. In agriculture, lanthanides

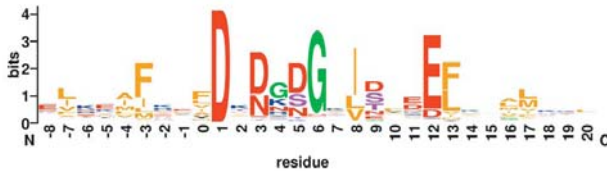

Figure 1. The sequence logo of the EF-hand domain [14]. The residues are colored as follows: D, E, N, red; K, R, blue; S, T, purple; G, green; hydrophobic, yellow.

have been used to increase the production of crops and to promote the growth of livestock in China for many years [15]. The molecular mechanism of the biological effects of lanthanides is not totally understood so far. Lanthanide ions (Ln^{3+}) have similar ionic radii and similar coordination properties to Ca^{2+} [16]. Hence, Tb^{3+} was usually used to sense properties of Ca^{2+}-binding proteins [17].

EoCen is the first reported by our laboratory [18] (gene register Y18899), which is cloned from *Euplotes octocarinatus*, and the detailed biological function is unclear. In this paper, in order to investigate the importance of the first amino acid (Asp37) of site I of EoCen to the metal-binding and conformation characteristics of this protein, the mutant protein (D37K), with the mutation of Asp37 to Lys, was obtained by the method of site direct mutation. Using TNS as fluorescence probe, the characterization for the binding of Tb^{3+} to EoCen and the mutant D37K were investigated.

2. MATERIALS AND METHODS

2.1. Reagents and Stock Solutions

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 purchased from Bio. Basic Inc. TNS was bought from Sigma. Glutathione Sepharose TM 4B (GST) was purchased from Pharmacia Ltd. Terbium oxide was 99.99% and purchased from Hunan in China. The terbium stock solution was prepared by dissolving weighed Tb_4O_7 in concentrated hydrochloric acid. The concentration of Tb^{3+} 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.

2.2. Protein Expression and Purification

Two proteins were used in this study, namely, EoCen (full-length of the wild type EoCen 1M-168Y), D37K (mutant EoCen with Asp37 changing to be Lys 1M-168Y). The D37K was acquired by polymerase chain reaction technique with p1 (5'-TATTTAAGACCAAC AAAACTGG-3') and p2 (5'-AATCAAAGGCTTCTTT GATCTC-3') used as primers. Proteins of EoCen were over-expressed off a PGEX-6p-1 plasmid construct in Escherichia coli BL21 (DE3) induced with isopropyl-Dthiogalactopyranoside (IPTG) to yield milligram quantities of the desired protein as reported previously [18]. Briefly, transformed E. coli cells were grown in LB media containing 100 μg/mL ampicillin and incubated at 37 ˚C while monitoring its growth via optical density (OD) measurements at 600 nm. Once OD600 reaches 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 g for 25 min at 4 ˚C. The supernatant was applied to a Glutathione-Sepharose TM 4B column which has been equilibrated with PBS buffer. After the initial purification by washing the supernatant with PBS buffer, prescissor proteinase was added at 4 ˚C for reacting about one night. Primary target proteins were washed out and eluted with PBS buffer. Then the proteins were applied to a superdex 75 column to be further purified. Fractions containing centrin were identified via 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After purification, the proteins were kept in -80 ˚C. The stock protein solutions were conserved in 10 mM Hepes.

2.3. Metal Lon Removal and Protein Concentration

To remove contaminating bound cation, the protein samples were ultrafiltrated extensively against 10 mM Hepes, pH 7.4 containing 1 mM EDTA. The protein concentration was measured spectrophotometrically using molar extinction coefficient at 280 nm of 5600 $L \cdot mol^{-1} \cdot cm^{-1}$. The extinction coefficient of centrin was estimated from the tyrosine content as previously reported [19].

2.4. Native- and SDS-PAGE Assays

Each protein sample with the same concentration was prepared in Hepes (10 mM, pH 7.4) for this experiment. Polyacrylamide gels contained 390 mM Tris (pH 8.8), 10% ammonium persulfate, 15% acrylamide/bis (29:1), and 0.1% TEMED. Tris-glycine electrophoresis running buffer contained 25 mM Tris (pH 8.3) and 250 mM glycine. All electrophoreses were run at room temperature. Gels were run at a constant current of 11-12 mA for 2 h. Gels were fixed in 50% methanol, 7% acetic acid for 1 h, washed in distilled water for 1 h, stained with Coomassie blue R-250 for 2 h, washed in distilled water for 2 h.

For SDS-PAGE, keeping same experimental conditions as above described except that 10% sodium dodecyl sulfate (SDS) was added and running buffer contained 25 mM Tris (pH 8.3), 250 mM glycine, 10% SDS was used.

2.5. Fluorescence Spectroscopy

The changes of the hydrophobic exposure degree of wild type EoCen and D37K were studied by monitoring the fluorescence emission spectra of the hydrophobic probe TNS. The fluorescence spectra of TNS were measured by Cary Eclipse spectrofluorometer (Varian Inc.). The excitation wavelength was set at 320 nm. The slit width of excitation and emission were both set at 10 nm. Fluorescence emission spectra were recorded with a single scan over the range 350-600 nm for TNS. The protein solutions were prepared by dilution of the stock solution with 10 mM Hepes pH 7.4 and 150 mM KCl. The TNS stock solution was gradually added to the mixed solution. The mixture were shaken thoroughly, and then equilibrated for 3 min at 25 °C in order to make the binding of TNS to protein was complete before measurements were taken.

3. RESULTS AND DISCUSSIONS

The effect of the mutation on the conformation of wild-type EoCen was monitored by Far-UV circular dichroism (CD), the Far-UV light CD spectra of EoCen and D37K are highly similar in shape (data not shown), which means that the mutation of aspartic acid does not disrupt the secondary structure of wild-type EoCen. Therefore, D37K was selected and purified for further research.

3.1. SDS- and Native-PAGE Assays

The mutant D37K had the same mobility as wild type EoCen on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (**Figure 2(a)**), since the molecular weight of D37K nearly did not change after mutation with an apparent molecular mass of 20 kDa, indicating highly purified proteins were obtained. **Figure 2(b)** indicates that on the native PAGE, the position of D37K is higher than that of WT-EoCen which is much closer to the bottom of the gel (the positive pole), exhibiting the much more positive net charge of D37K that is obviously different from the SDS-PAGE (mutation of Asp37 does not disrupt the secondary structure shown in CD spectra), indicating that the mutate D37K is surely obtained.

3.2. The Conformation Change of Proteins

2-p-toluidinylnaphthalene-6-sulfonate (TNS) is a useful probe of conformational changes, because its fluorescence is altered when it binds to hydrophobic patches on the accessible surface of proteins [8,20,21]. Undergoing a large conformational change is required for the trigger proteins (Ca^{2+}-modulated proteins or sensor proteins such as CaM and troponin C) to regulate a vast number of target proteins [22,23]. As shown in **Figure 3**, TNS had a weak fluorescence in water alone, and the fluorescence intensity increased largely with binding to EoCen and Tb^{3+}-saturated EoCen, accompanied by a blue shift of the maximum fluorescence peak from 500 to 435 nm, indicating the probe transferred from the polar to the apolar environment and the Tb^{3+}-saturated centrin exposed more hydrophobic surface. **Figure 4** is a set of fluorescence spectra caused by the addition of Tb^{3+} (3.35×10^{4}M) solution to 1.5 mL WT-EoCen (8 μM) in the presence of TNS in 10 mM Hepes at pH 7.4, 150 mM, 25 °C. The plot of the fluorescence intensity of TNS at 435 nm versus [Tb^{3+}]/[protein] is shown as **Figure 5**.

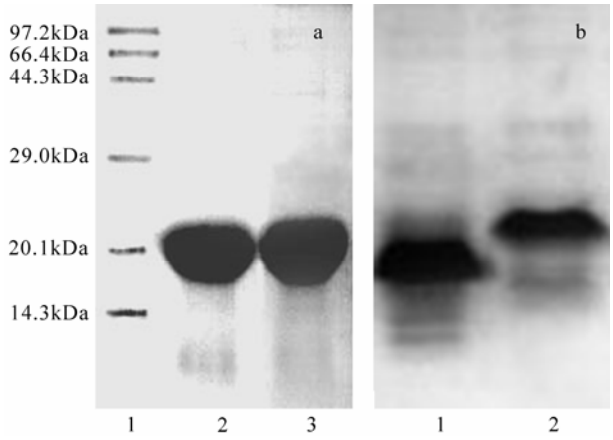

Figure 2. (a) The purified wild type apoEoCen and D37K monitored by 15% SDS-PAGE. Lane 1, Mr, molecular size marker; Lane 2, WT-EoCen; Lane 3, D37K. (b) The purified wild type apoEoCen and D37K monitored by 15% native PAGE. Lane 1, WT-EoCen; Lane 2, D37K.

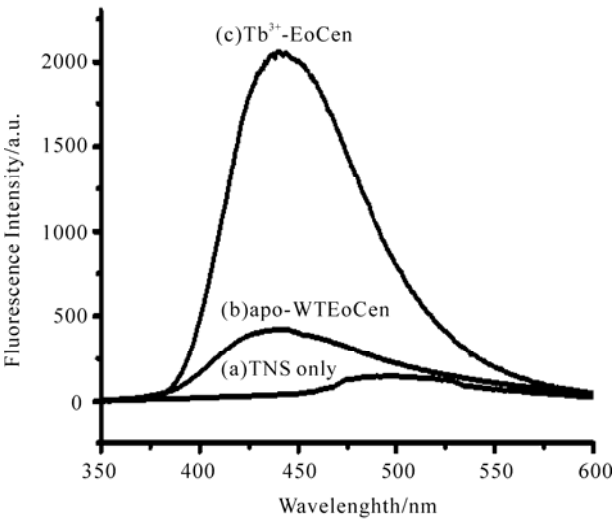

Figure 3. Fluorescence spectra of TNS alone (a); in the presence of apoEoCen (b); and Tb^{3+}-saturated WT-EoCen (c). The protein concentration is 8 μM in 10 mM Hepes, 150 mM KCl, at pH 7.4 and 25°C.

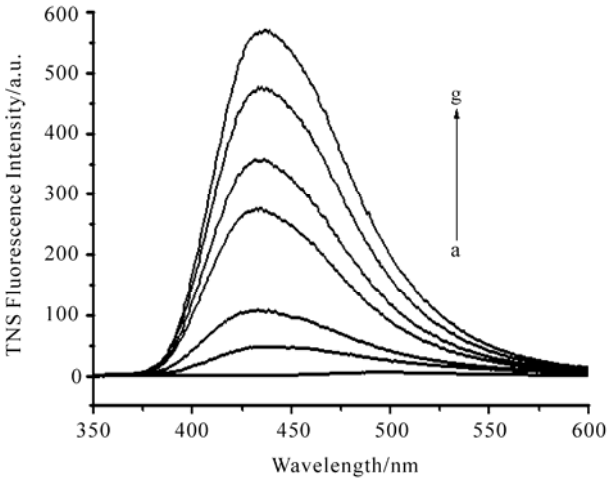

Figure 4. Fluorescence spectra of Tb^{3+} binding to EoCen in the presence of TNS. (a) $[Tb^{3+}]/[P]=0$; (b) $[Tb^{3+}]/[P]=1.0$; (c) $[Tb^{3+}]/[P]=2.0$; (d) $[Tb^{3+}]/[P]=2.5$; (e) $[Tb^{3+}]/[P]=3.0$; (f) $[Tb^{3+}]/[P]=3.5$; (g) $[Tb^{3+}]/[P]=4.0$. The protein concentration is 8 μM in 10 mM Hepes, 150 mM KCl, at pH 7.4 and 25 °C.

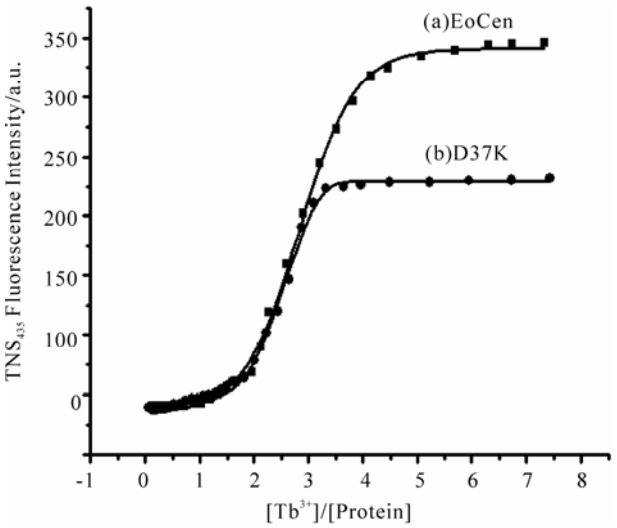

Figure 5. Titration curves of the addition Tb^{3+} to the WT-EoCen and D37K in the presence of TNS, in 10mM Hepes, pH 7.4, 25 °C. The protein concentration is 8 μM.

It can been seen from **Figure 4** that there is a peak at near 435 nm, and which increase obviously with the addition of metal ions, when the value of $[Tb^{3+}]/[protein]$ is equal to near 4, the increase become very slow (**Figure 5**, curve (a)). The conclusion, the affinities of sites IV and III (C-terminal domain) with Tb^{3+} are higher than that of sites I and II (N-terminal domain), has already obtained [24]. From **Figure 4**, the results show that EoCen undergo a conformational change when binding the Tb^{3+}, and the change extent of hydrophobic exposure between the C-terminal domain and the N-terminal domain is different and the N-terminal domain is larger than the C-terminal domain, induced by metal ions (**Figure 5**). So the C-terminal domain and N-terminal domain play a distinct role in the process of centrin realizing itself biological function. This is a possible explanation of that N-terminal of centrin responsible for self-assembly, while C-terminal serves as recognizing the target protein or enzyme, which is accord with the reports previously [25,26]. It can be seen from **Figure 5** that D37K can only bind three equivalents Tb^{3+} (WT-EoCen binds four equiv. Tb^{3+}). When Asp37, the first amino acid of loop I, was mutated to lysine, the net charge of loop I (in D37K) is changed from -1 to +1. Due to the electrostatic repulsion between the positively charged loop and Tb^{3+}, the metal-binding ability of loop I in D37K is abolished. TNS fluorescence intensity induced by the Tb^{3+} binding to D37K is obviously declined. It can be concluded that the mutation of Asp to Lys at position 37 leads to the smaller exposure of hydrophobic surface in the presence of Tb^{3+}, which can be attributed to the fact that the ability of metal-binding of loop I was abolished by mutation of Asp37 to lysine. It proved that the Asp37 plays an important role in maintaining the proper conformation of EoCen in the presence of Tb^{3+}.

3.3. The Calculation of the Binding Constants of TNS Interaction with Tb^{3+}-Loaded Proteins

Assuming that there are *n* TNS-binding sites and they are independent and identical in TNS-protein complex, the conditional binding constants [27-29] can be fitted using **Eqs.1-6** from the data of curves a and b in **Figure 6**.

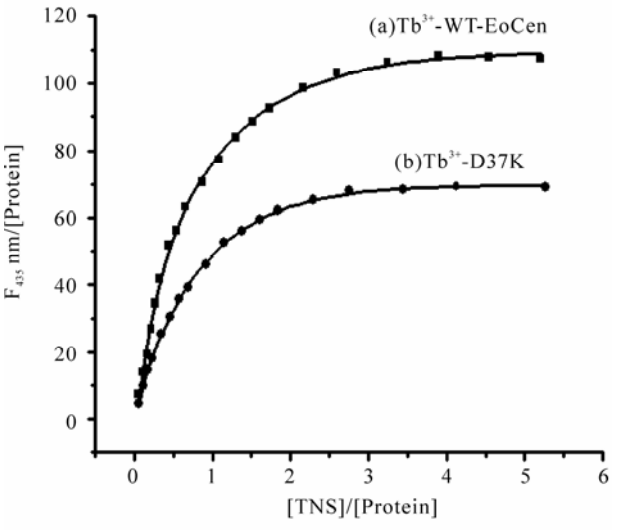

Figure 6. Titration curves of the addition TNS to the different form of proteins, in 10 mM Hepes, 150 mM KCl, pH 7.4, 25 °C. (a) Tb^{3+}-saturated WT-EoCen, (b) Tb^{3+}-saturated D37K. The protein concentration is 8 μM.

The binding equation is presented by:

$$n\mathrm{TNS} + \mathrm{P} \leftrightarrow \mathrm{TNS}_n\mathrm{P} \quad (1)$$

Given that 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:

$$F_\infty \propto n[\mathrm{P}]_t \quad (2)$$

$$F_r \propto [\mathrm{TNS}]_b \quad (3)$$

$$[\mathrm{TNS}]_b = \frac{F_r \cdot n[\mathrm{P}]_t}{F_\infty} \quad (4)$$

where $[TNS]_b$ is the bound concentration of TNS. $[P]_t$ and $[P]_b$ is the total and bound concentration of protein in 10 mM Hepes, pH 7.4, 25 °C, respectively. The binding constant can be given as follows:

$$K = \frac{[\mathrm{TNS}]_b}{[\mathrm{TNS}]_f \cdot n[\mathrm{P}]_f} \quad (5)$$

$[TNS]_f$ and $[P]_f$ represent the free concentration of TNS and protein, respectively. Finally, equation can be expressed by **Eq.6**.

$$\frac{[\mathrm{TNS}]_t}{[\mathrm{TNS}]_b} = 1 + \frac{1}{K\{n[\mathrm{P}]_t - [\mathrm{TNS}]_b\}} \quad (6)$$

For Tb^{3+}-EoCen, 1/K can be obtained by the linear slope of the plot of $[TNS]_t/[TNS]_b$ versus $\{[Tb^{3+}\text{-EoCen}]_t - [TNS]_b\}^{-1}$, as showed in **Figure 7**. *n* is set to be 1, and the intercept of **Eq.6** is very close to 1, and can also be seen in **Figure 6**, *n*=1 is suitable. K (Tb^{3+}-EoCen-TNS) can be calculated to be (7.38±0.25) $\times 10^5 M^{-1}$ from **Figure 7**. In the similar way, K (Tb^{3+}-D37K-TNS) can be calculated to be (1.16±0.05) $\times 10^6 M^{-1}$.

It can be seen that the affinities of TNS binding to EoCen and D37K are different, K (Tb^{3+}-D37K-TNS)>K (Tb^{3+}-EoCen-TNS), showing that D37K, with more positive charges, has larger electrostatic interaction with TNS than EoCen in the presence of Tb^{3+} and the mutation of Asp37 influence the hydrophobic and electrostatic environment of WT-EoCen.

4. CONCLUSIONS

Using the fluorescence spectroscopy, the interaction of TNS and EoCen has been studied in the presence of Tb^{3+}. WT-EoCen and D37K undergo a different extent conformation change, and D37K exposes lesser hydrophobic surface in the presence of Tb^{3+}, which can be attributed to the fact that the ability of metal-binding of site I was abolished by the mutation of Asp37 to lysine. It can be concluded that the Asp37 plays an important role in maintaining the proper conformation of EoCen in the presence of Tb^{3+}. In the end, the binding constants of TNS with Tb^{3+}-saturated EoCen and D37K were obtained, K(Tb^{3+}-EoCen-TNS)=(7.38±0.25)$\times 10^5 M^{-1}$ and K(Tb^{3+}-D37K-TNS)=(1.16±0.05)$\times 10^6 M^{-1}$.

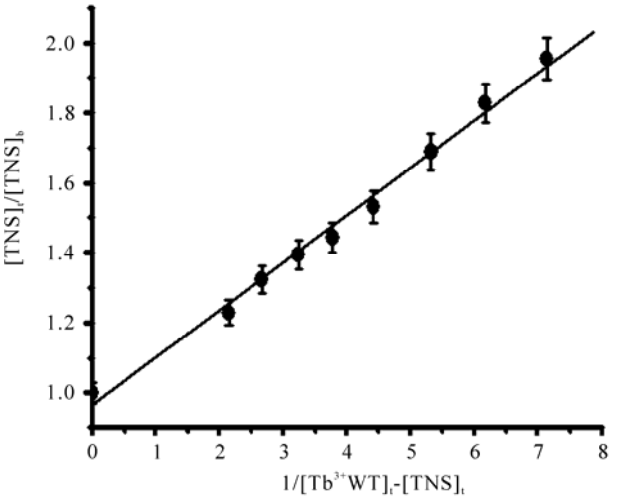

Figure 7. The plot of $[TNS]_t/[TNS]_b$ vs $\{[WT\text{-EoCen-Tb}]_t\text{-}[TNS]_b\}^{-1}$.

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