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Tb(III) AND Eu(III) AS FLUORESCENT PROBES TO INVESTIGATE THE METAL-BINDING SITES OF TRICHOSANTHIN+

Yan Zhang[1], Wenlian Li*[2], Qiang Hao[3], Gui Yu[2], Qingshan Li[1] and Qizhi Yao[3]

[1] Department of Molecular Biology, JiLin University, Changchun 130023, China

[2] Changchun Institute of Physics, Academia Sinica, Changchun 130021, China

[3] Graduate School of Academia Sinica, Beijing 100039, China

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Tb(III) and Eu(III) ions were used as fluorescent probes in the study of the trichosanthin (TCS). The fluorescence of Tb(III) was increased considerably when bound to TCS to replace the Ca(II) ions. The nonradiative energy transfer from fluorescent tryptophan (Trp) residues to the bound Tb(III) or Eu(III) took place. From a Foster d-d nonradiative energy transfer mechanism, it was obtained that the average distance between the bound Tb(III) and the Trp residue is 1.27nm. The results indicate that the major groups in TCS bound to Ca(II) ions should be the carboxylic side groups of the glutamic acid and / or aspartic acid. The fluorescent quenching of Ca(II)-free TCS by adding Tb(III) or Eu(III) into TCS from which the Ca(II) ions had been removed was discussed as well.

Many active proteins require metal ions for their activities or contain bound metal ions for their native structure and function. Some of the metal ions exhibit useful spectroscopic or magnetic probe properties, while others, e.g., Ca(II), Mg(II) do not. When the native metal ions is devoid of useful

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* To whom correspondence should be addressed.

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properties , it is often possible to substitute a metal ion with useful characteristics for detecting it . The trivalent lanthanide ions , Ln(Ⅲ), have been used as fluorescent probes to replace Ca(Ⅱ), Mg(Ⅱ) and so on in a variety of proteins [1-3], and without changing significantly the structure of the protein molecules. Thus, with studying the fluorescence of Ln(Ⅲ) and the energy transfer between Ln(Ⅲ) and some amino acid residues of proteins, some information about the structure and function of protein can be obtained.

The structure of bovine serum albumin (BSA) was quantitatively studied with Tb(Ⅲ) fluorescent probes in our laboratory [4]. In this paper, we report the results on the structure of another important Ca(Ⅱ)- containing protein - TCS by Ln(Ⅲ) fluorescent probes. TCS belongs to the family of single-chain ribosome inactivating proteins (RIP), which inhibit the translation of gene. TCS was purified from the dried root slice of *Trichosanthes Kirilowii*, the chinese medicinal herbs , which was first used as an abortifacient drug of midgestation[5]. It was found that TCS can inhibit HIV, as the etiologic agent for AIDS in 1987 [6], and have enzymatic activities of glycosidase and DNAase [7]. The molecular weight of TCS is about 27, 000 Dalton , and is composed of 247 amino acid residues in which only one tryptophan residue exsits at position 192 [8].

Materials And Methods.

Materials: 99.99% of Tb_4O_7 and Eu_2O_3 were purchased from Baotou Institute of Rare Earth, China. The other reagents used in the experiments were all analytical grade. Doubly distilled and deionized water was used throughout.

Instruments: Fluorescence spectra were recorded on a Hitachi Fluorescence Spectrophotometer, Model F-4000. UV absorption spectra were determined on a Shimadzu Dual-Wavelength / Double-Beam Recording Spectrophotometer, Model UV-250. Absorbance measurements at single wavelengths were made with a Shimadzu Single-Beam Spectrophotometer, Model UV-120.

Purification and Characterization of TCS: The dried root slice of *T. Kirilowii* was obtained in a local drugstore. All operations were performed at 4℃ . The crude homogenate with 0.05M Tris-HCl buffer at pH 6.8 (buffer A), homogenized with a high-speed blender, and then centrifuged in a refrigerated centrifuge . Ammonium sulphate of 40% (w/v) saturation was added to the collected supernatant. The mixture was left for 12 hours and centrifuged. The collected supernatant was adjusted to 75% (w/v) saturation with the

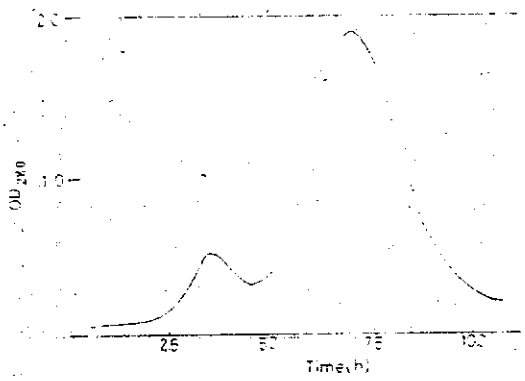


Fig. 1. Application of crude TCS from CM-Sepharose chromatography to Sephadex G-75. The main peak was found to contain a single band at 27 KDa on SDS-PAGE (7-20% gradient gel).

ammonium sulphate, left for 6 hours, and centrifuged. The collected precipitate was resuspended with water, and dialyzed overnight against water and buffer A. There sulting solution was loaded onto a DEAE-cellulose column equilibrated with buffer A, then eluted with buffer A. The eluting peak was applied to a CM- sepharose column, washed with buffer A, and eluted with buffer A containing 0.3M NaCl, respectively. The second peak was collected, and put it onto the third column of Sephadex G-75 which is equilibrated with buffer A, and eluted under the same conditions. The second peak, as shown in Fig. 1, was collected, and lyophilized. Purity determination of Sephadex G-75 purified trichosanthin on SDS-PAGE (9～20% gradient gel) was revealed as a single band with M. W. 27KDa.

In the whole procedure UV absorptions at 260nm and 280nm were used to examine the exist and quantity of proteins. The activity was measured by the immunodiffusion method using anti-TCS antibody [9].

Protein concentrations were determined from absorbance of TCS at 280nm by using ε_{280}=18, 800$M^{-1}cm^{-1}$ [8].

Ca(II)-free TCS was obtained by using EDTA based on the method of shown by Henzl, M. T. et al [11].

Preparation of Tb(III) and Eu(III) ions : preparation of Tb(III) and Eu(III) ions is the same as the method described in Ref. 4. The concentration of Tb(III) and Eu(III) was determined by EDTA titration using xylenolorange as the end point indicator. The EDTA was standardized with Zinc oxide.

Results And Discussion

1. Energy transfer between the tryptophan and the metal ion in TCS molecules

The fluorescent emission spectra of TCS in the presence or absence of Ln(III) ions were determined by excitation at 295nm , as shown in Fig. 2. The effect of tyrosine on the spectra can avoided in this condition [10]. From Fig. 2, it seems that the peak at 335 nm in the spectra should be attributed to tryptophan. It was also noticed that when Tb(III) or Eu(III) ions were added into the TCS solution, respectively, the fluorescence intensities of the tryptophan were effectively decreased, up to 79% by Tb(III), up to 64% by Eu(III). The quenching effect by the Ca(II) ions, however, was almost not observed. Therefore, it can be concluded that the energy transfer from the tryptophan residue upon the binding Tb(III) or Eu(III) took place, but the Ca(II) ions do not cause the similar effect.

The emission spectra of Tb(III) are shown in Fig. 3, which reveals four characteristic Tb(III) fluorescent peaks between 480-639 nm . Among the quartat, the emission peak at 545 nm corresponding to $^5D_4 \rightarrow {}^7F_5$ transition of the Tb(III) is most sensitive to the environmental change . From Fig. 3 it is seen that the emission peak at 545nm is gradually increased by the sequence: aqueous Tb(III), Tb(III)-TCS complex, Tb(III)-TCS[Ca(II)-free] complex.

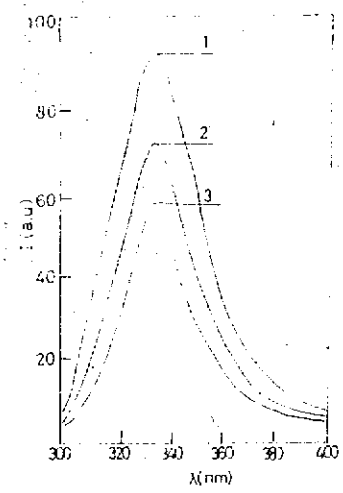
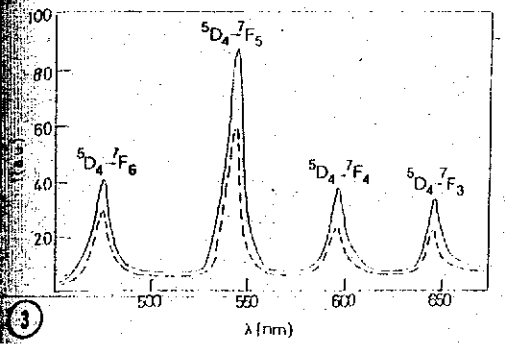


Fig. 2. Fluorescence spectra of Trp of TCS.
(1) TCS (2) TCS-Tb(III) complex (3) TCS-Eu(III) complex. Excitation: 295 nm, TCS: 3. 7×10^{-6}M, Tb(III) or Eu(III): 5×10^{-3}M, pH 6.3, in hexamethylene-tetramine buffer, ionic strength 0. 1M.



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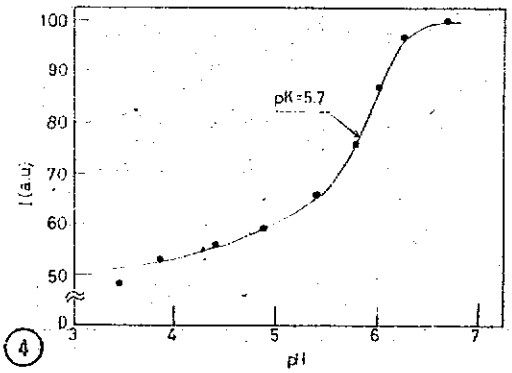

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Fig. 3. Fluorescence emission spectra of Tb(Ⅲ)(……), Tb-TCS complex(— · —) and Tb(Ⅲ)-TCS(Ca(Ⅱ)-free) complex(——). Excitation: 295nm. Tb(Ⅲ): 5×10^{-5}M, TCS or TCS(Ca(Ⅱ)-free): 3.7×10^{-6}M.

Fig. 4. pH dependence of the relative emission intensities of fluoresrence of TCS-Tb(Ⅲ) complex at 545 nm. TCS: 3.7×10^{-6}M, Tb(Ⅲ): 5×10^{-5}M.

We can conclude that nonradiative energy transfer occurs from Trp residue to the bound Tb(Ⅲ). For Ca(Ⅱ)-free TCS, Tb(Ⅲ) ions can easily enter the metal-binding sites in TCS, because there is not the competing bound Ca(Ⅱ) (that results in energy transfer from tryptophan to the bound Tb(Ⅲ) ion). So the fluorescent intensities is stronger in the Ca(Ⅱ)-free TCS than in the TCS. It is also implied that when Ca(Ⅱ) was removed from TCS, there should still exist metal binding sites in TCS molecules.

The enhancement of Eu(Ⅲ) fluorescence upon binding to TCS, however, was not observed for neither the absence nor the presence of Ca(Ⅱ) ion in TCS. The lack of enhancing emission in the case of Eu(Ⅲ) causes from charge transfer band [11]. Thus, the energy absorbed by TCS was markly transferred to the Eu(Ⅲ), and considerably all radiationlessly lost, and then does not appear as luminescence.

2. Stability of TCS-Tb(Ⅲ) complex as a function of pH

Stock solutions of TCS containing $TbCl_3$ were adjusted close to the desired volume with an unbuffered aqueous solution at pH 6.3. The pH of each solution was then adjusted with dilute HCl to the required pH value, and emission intensities of the Tb(Ⅲ) ion were then measured. The titration curve was shown in Fig. 4. It is shown that the curve is similar to "S" type.

the relative high Tb(Ⅲ) emission was o... ...ven at low pH value(pH 2-4), which doesn't seem to like the results reported in Ref. 4. The curve (Fig. 4) also shows the pK of the sigmoidel titration curve is 5.7 , which implies the apparent pK of equilibrium (1) is 5.7.

$$Tb^{3+}+protein-(COOH)_n=protein-(COOH)_nTb^{3+}+nH^+ \quad (1)$$

This equilibrium is the sum of two equilibria

$$protein-(COOH)_n=protein-(COO-)_n+nH^+ \quad (2)$$

$$protein-(COO-)_n+Tb^{3+}=protein-(COO-)_nTb^{3+} \quad (3)$$

When pH value is smaller than 5, although the carboxylic groups might be ionizated in the equilibrium system, Tb(Ⅲ) ions are not necessarily bound to the carboxylic groups in TCS. When pH value is up to 5.7, the Tb(Ⅲ) fluorescence was fairly enhanced, amount of the bound Tb(Ⅲ) ions is increased. It indicates that the carboxylic groups in TCS must be at ionized state . Since only carboxylic side chains of aspartic and / or glutamic acid residues can be ionized at pH value lower than 5. 7(pK=3.8 for Asp-β-COOH , pK=4.2 for Glu-γ-COOH), so we can conclude that the ligands are carboxylic side chains of these two amino acid residues.

3. Distance Between the Bound Tb(Ⅲ) and Trp Residue in TCS molecule

Based on Forster's theory, the efficiency of energy transfer, E, from a donor to an accpetor is related to the actual distance of separation, and the distance for 50% energy transfer, R_0 by eq. (4), R_0 is defined by eq. (5) [12].

$$E=1+[(r/R_0)^6]^{-1} \quad (4)$$

$$R_0^6=8.\ 78\times10^{-25}K^2\phi_{Trp}n^{-4}J. \quad (5)$$

where ϕ_{Trp} is the quantum yield of the donor Trp in the absence of acceptor, and n is the refractive index of the medium between the donor and acceptor, J is the spectral overlap intergral defined by

$$J=\frac{\int F(\upsilon)\varepsilon(\upsilon)\upsilon^{-4}d\upsilon}{\int F(\upsilon)d\upsilon}, \quad (6)$$

where $F(\upsilon)$ is the fluorescence intensity of the donor, (υ) is the molor extinction cofficient of the acceptor in units of $(mol/L)^{-1}cm^{-1}$, and υ is the frequency in cm^{-1}.

Tb(Ⅲ)-acetic acid complex was used as a model and J value of 0.738×$10^{-19}cm^{6}(mol/L)^{-1}$ was found in our group. R value calculated from these data is 0.346 nm [4].

E is given by

$$E = \frac{A_{Tb(III)}\Phi_{Trp}}{A_{Trp}\Phi_{Tb(III)}}, \qquad (7)$$

where $A_{Tb(III)}$ and A_{Trp} are the integrated areas of fluorescence emisson of Tb(Ⅲ) and Trp in the protein. From the experiment, we determined that the value of $A_{Tb(III)}/A_{Trp(III)}$ is 8.2×10^{-4}, Φ_{Trp} and $\Phi_{Tb(III)}$ are the quantum yields of the Trp and Tb(Ⅲ) in the protein. Using Φ_{Trp}=0.14[13] and $\Phi_{Tb(III)}$ =0.28 [14], then the value of E is found to be 4.3×10^{-4}. If we put the value of E and R_0 of 4.3×10^{-4} and 0.[illegible] nm into eq. (4), respectively, then 1.27 nm of r value was determined. That is, the distance between Trp 192 of TCS and its bound Tb(Ⅲ) ion and should be regarded as that between the Trp and its bound Ca(Ⅱ) ion in natural state.

Acknowledgments

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