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TB(III) AS A FLUORESCENT PROBE FOR THE STRUCTURE OF BOVINE SERUM ALBUMIN

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Tb(III) was used as a fluorescent probe in the study of the calcium-binding sites on Bovine Serum Albumin(BSA). The fluorescence of Tb(III) is enhanced markedly when bound to BSA and nonradiative energy transfer between two fluorescent tryptophan(Trp) residues and Tb(III) bound to calcium-binding sites on BSA occurred. Experimental results show that the major groups in BSA bound to metal ion are the carboxyl side groups of glutamic acid(Glu) and aspartic acid(Asp). The average distance between the bound Tb(III) and the two tryptophan residues in BSA calculated by a Föster dipole-dipole nonradiative energy transfer mechanism is 1.48 nm.

Many proteins either contain Ca(II) as a basic structural and functional entity or utilize this ion in carrying out their biological function. In contrast to proteins bearing transition metal ion, those involving Ca(II) are considerably more difficult to investigate by spectroscopic techniques. The electronic transition of Ca(II) cannot be studied by conventional optical absorption and emission spectroscopy, and the absence of unpaired electrons precludes the use of magnetic resonance techniques in probing the chemical and structural nature of calcium ion binding sites.

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Effective ionic radii of Tb(III) is almost equal to that of Ca(II). It suggests that Tb(III) may be used to bind to Ca(II)-binding sites without causing serious structural modification. Perhaps of greater importance to the potential use of Tb(III) ion as a probe for calcium-binding sites on BSA is the strong propensity of both Tb(III) and Ca(II) ions for oxygen donor groups.

BSA is one of major components in the plasma protein, and it can combine with some substances with low molecular weight in blood for controlling their concentrations, including that of calcium ion. BSA, whose molecular weight is 68,000, is composed of 582 amino acid residues in which two tryptophan residues exist at position 134 and 212, respectively. BSA comprises three domains[1] and each domain combining with different small molecules leads to the corresponding comformational change of BSA. In this paper, we used Tb(III) as fluorescent probe to study the calcium-binding sites on BSA and measurments on energy transfer were involved.

Experimental

Materials: BSA was the product of Shanghai Institute of Biochemistry and was used without further purification. 99.9% of Tb_4O_7 was purchased from Baotou Institute of Rare Earth. The stock solution of Tb(III) was prepared in the 0.1 M concentration range from Tb_4O_7. Tb_4O_7 was dissolved in more than 12 equiv of hydrochloric acid. The solution was mixed under constant moderate heating until water and extra hydrochloric acid were all eliminated.

Terbium ion concentrations were determined by EDTA titration using xylenol orange as the end point indicator. The EDTA was standardized using ZnO. The other chemical reagents used were all of analytical grade. Doubly distilled deionized water was used throughout.

The emission experiments were conducted at pH6.2 in a hexamethylene-tetramine buffer at 0.1 M ionic strength controlled with KCl. At pH6.2 hydroxy complexes of Tb(III) do not appear and Tb(III) does not complex

to the buffer which contains only nitrogen donor atoms. Ethanol was used as the solvent for the Tb(III)-Acetic acid complex for U.V. absorption spectrum measurment.

Instrumentation: Fluorescence spectra were recorded on a Hitachi Fluorescence Spectrophotometer, model F-4000 and U.V. absorption spectra were determined on a Shimadzu Dual-Wavelength/Double-Beam Recording Spectrophotometer, model UV-3000. The two instruments all have the automatic compensation and calibration systems.

Results and Discussion

1. Enhancement of Tb(III) Fluorescence upon Binding to BSA. Fig.1 shows that the fluorescence enhancement of the Tb(III) was brought about by BSA when excited at 295 nm. At 295 nm, the protein emission is due to tryptophan without contribution of tyrosine.[2] The spectrum reveals the protein fluorescence peak at 348 nm belonging to tryptophan and the characteristic Tb(III) fluorescence quartat between 480 and 630 nm. Among the quartat, the emission peak at 545 nm corresponding to $^5D_4 \rightarrow {}^7F_5$ transition of Tb(III) is most sensitive to the environmental change. From Fig.1 we can conclude that nonradiative energy transfer occurs from Trp residues to the bound Tb(III).

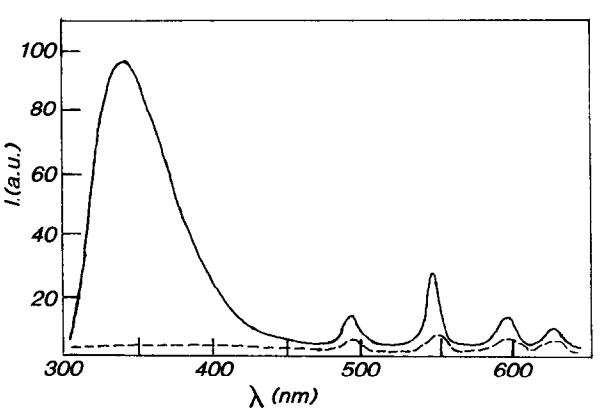

Figure 1.
Fluorescence spectra of 5.0×10^{-2} M Tb(III) in the absence of BSA in hexamethylene-tetramine buffer, pH6.2(----), and in hexamethylene-tetramine buffer, pH6.2(———), containing 5.0×10^{-6} M BSA.

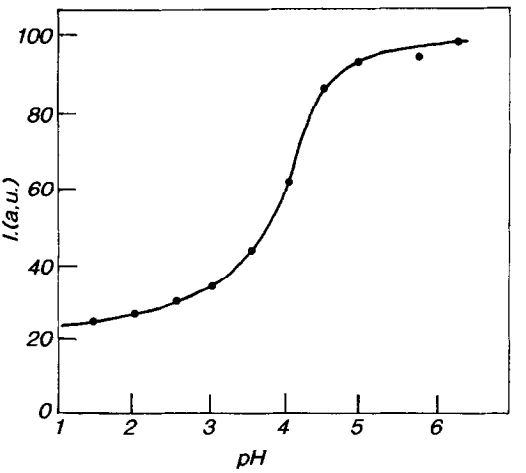

Figure 2.

Dependence on pH of the fluorescence of Tb(III)-BSA complex at 545 nm. BSA, 5.0×10^{-5} M; Tb(III), 5.0×10^{-3} M.

2. The Stability of the Tb(III)-BSA Complex as a Function of pH. The pH dependence of the Tb(III)-BSA complex formation was measured. Stock solution of BSA and $TbCl_3$ were added together and adjusted to the final volume with an unbuffered aqueous solution at pH6.2. The pH of each solution was then adjusted with dilute hydrochloric acid to the desired value and the fluorescence intensity of the Tb(III) band at 545 nm was subsequently measured. The results(Fig.2) show a sigmoidal titration curve with an apparent pK of 4.1. Because the pK values of the carboxyl groups of Asp and Glu residues in protein are between 3.0 and 4.7[3], one can safely conclude that the metal ligands are the carboxyl side chains of Asp and Glu residues.

3. Distance Between the Bound Tb(III) and Tryptophan Residues. According to Förster, the efficiency of energy transfer, E, between a donor and acceptor is related to the actual distance of separation, r, and the critical distance for 50% energy transfer, R_o, by eq.(1). R_o is defined by eq.(2),

$$E = \left[1+(r/R_o)^6\right]^{-1} \quad (1)$$

$$R_o = 8.78 \times 10^{-25} k^2 \phi_{rp} n^{-4} J \quad (2)$$

ϕ_{Trp} is the quantum yield of the donor tryptophan in the absence of acceptor, and n is the refrative index of the medium. J is the spectral overlap integral defined by eq.(3),

$$J = \frac{\int F(\upsilon)\varepsilon(\upsilon)\upsilon^{-4}d\upsilon}{\int F(\upsilon)d\upsilon} \qquad (3)$$

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

In Fig.3, we used Tb(III)-Acetic acid complex as a model and found J to be $0.738 \times 10^{-19} cm^6 (mol/L)^{-1}$. In the valution of R_o, k^2 value of 2/3, n value of 1.33 for experimental medium being dilute solution and ϕ_{Trp} value of 0.14[4] were taken. The R_o value calculated from these data is 0.346 nm. E is given by eq.(4),

$$E = (A_{Tb(III)}/A_{Trp}) \cdot (\phi_{Trp}/\phi_{Tb(III)}) \qquad (4)$$

where $A_{Tb(III)}$ and A_{Trp} are the integrated areas of fluorescence emission of Tb(III) and Trp in the protein, respectively, and $\phi_{Tb(III)}$ and ϕ_{Trp} are the respective quantum yield. Taking $\phi_{Trp} = 0.14$[4] and $\phi_{Tb(III)} = 0.28$[5]

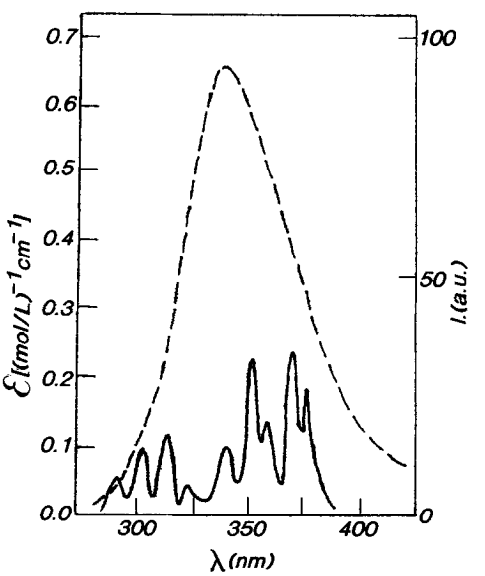

Figure 3.

Spectra overlap between the absorption spectrum of a 1:3 Tb(III)-Acetic acid complex (———) and the fluorescence emission of tryptophan from BSA (– – – –).

with the experimentally determined value for ($A_{Tb(III)}/A_{Tyr}$) of $3x10^{-4}$, one gets $E= 1.62x10^{-4}$. Finally, values of E and R_o of $1.62x10^{-4}$ and 0.346 nm, respectively, lead to an r value of 1.48 nm by eq.(1). Conveniently, we can deduce the comformational change of BSA bound to small molecule from variations of r value using Tb(III) as fluorescent probe.

Acknowledgments

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