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FLUORESCENCE OF PHOSPHOTYROSINE - TERBIUM(III) COMPLEXES

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Summary: Phosphotyrosine, a biologically important protein residue, was investigated for the ability to enhance terbium (Tb^{3+}) fluorescence. Spectroscopic analysis of the Tb^{3+} : phosphotyrosine interaction indicated the development of a new excitation peak at 275 nm and strong Tb^{+} fluoresence enhancement at 488 and 540 nm that was linear over a range from 0.5 to 100 μM amino acid. Subsequent experiments comparing the ability of phosphotyrosine, phosphothreonine, phosphoserine and 20 other common non-phosphorylated amino acids showed that only phosphotyrosine produced significant Tb^{3+} fluoresence enhancement. Analysis of various phospho-sugars and nucleotides showed (with the expected exception of GMP) that they produced little or no significant fluoresence enhancement, indicating a further selectiveness for the phosphotyrosine : Tb^{3+} fluoresence enhancement event. These results establish a basis for the future use of Tb^{3+} fluoresence enhancement as a unique probe for the investigation of phosphotyrosine residues.

Previous studies have demonstrated that the natural fluorescence of the terbium trivalent cation (Tb^{3+}) can be enhanced several hundred to several thousand fold through interaction with specific UV-light-excited, aromatic residues of both proteins and nucleic acids. For example, Tb^{3+} has been shown to be an effective probe for the spectroscopic detection and study of Ca^{2+} binding sites in proteins (1). In these studies, Tb^{3+} was able to replace Ca^{2+} in protein calcium binding sites and undergo fluorescence enhancement due to an unexpected high frequency of nearby polypeptide aromatic sidechains capable of UV-light absorption and energy transfer to Tb^{3+} for subsequent emmission. In other studies involving Tb^{3+} fluorescence enhancement by nucleic acids, Tb^{3+} was shown to be a specific probe of guanine residues residing in single strand regions of DNA and RNA (2-4). In this case, the mechanism involved the ability of Tb^{3+} to bind to phosphates of the nucleic

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acid phosphodiester backbone in close proximity to an aromatic guanosine residue which, again, when appropriately UV-irradiated, transferred excitation energy to Tb^{3+} for fluorescence emission.

Tyrosine phosphorylation is unique among protein post-translational modifications because of its tight linkage with peptide growth hormone signal transduction and the transforming proteins of retroviral oncogenes (see refs. in 5). The overall level of phosphotyrosine residues in biological tissues is relatively low, e.g., 0.02 to 0.06% of the acid-stable protein bound phosphate (6). Typically, the study of phosphotyrosines have required the use of [P^{32}]-labeling techniques (6) or the use of phosphotyrosine antibodies (7) for detection. Since the structure of phosphotyrosine is comprised of an anionic phosphate for binding Tb^{3+} in close proximity to an aromatic phenol ring capable of UV-absorption, it was considered a candidate for producing Tb^{3+} fluorescence enhancement. Results reported herein demonstrate that phosphotyrosine acts as a strong enhancer of Tb^{3+} fluorescence and moreover does so in a highly selective manner suitable for future use in the study of phosphotyrosine residues.

MATERIALS AND METHODS

Tb^{3+} stocks were made from $TbCl_3{:}6H_2O$ (99.99%) supplied by Alfa Products, Danvers, MA. Tyrosine-O-sulfate was a generous gift from Dr. M.-C. Liu, Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma. All other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri.

Enhancement of Tb^{3+} fluorescence was determined essentially as described previously (3). Measurements were made with an Aminco-Bowman recording spectrophotometer equipped with an Aminco photon counter. The fluorescence readings were reported from spectra uncorrected for instrumental characteristics and were given in units of relative fluorescence. Standard solutions of quinine sulfate (18 μM) used to monitor instrument variation during data collection. Fluorescence enhancement levels were calculated by subtracting the fluorescence of control samples containing Tb^{3+} alone from experimental samples containing Tb^{3+} and a test compound, e.g., phosphotyrosine. A typical experimental sample contained 20 mM cacodylate (pH 6), 10 mM KCl, 10-800 μM Tb^{3+}, and 0.5-800 μM test compound. Samples were allowed to equilibrate 10 min at room temperature before fluorescence determinations and all fluorescence measurements were made at room temperature (22°C).

RESULTS

Phosphotyrosine enhancement of Tb^{3+} fluorescence. It has been previously demonstrated that Tb^{3+} fluoresces with emission maxima at 488 and 540 nm when excited at appropriate wavelengths and when present in concentrations of 1 mM or higher (2). Enhancement of Tb^{3+} fluorescence by other compounds can be conveniently determined at lower Tb^{3+} concentrations where contributions to fluorescence from free Tb^{3+} is so low as to be insignificant. The ability of

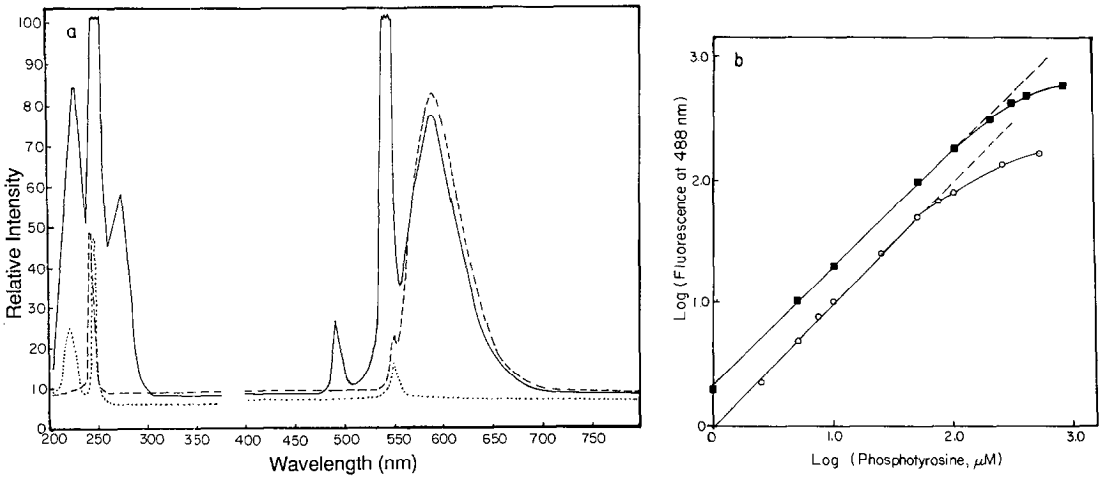

Figure 1. Enhancement of Tb^{3+} fluorescence by phosphotyrosine. (a) An excitation (λem 488 nm) and emission (λex 275 nm) spectrum of 100 μM Tb^{3+} (•••), 100 μM phosphotyrosine (---), and 100 μM Tb^{3+} plus 100 μM phosphotyrosine (—). (b) Enhancement of 100 μM (O) or 400 μM (■) Tb^{3+} by 0.5 to 800 μM phosphotyrosine. Conditions for fluorescence determinations are given in Materials and Methods. Each point is the mean of 3 or more independent determinations, standard errors about the means were smaller than the graphical space occupied by the data points.

Tb^{3+} to display fluorescence enhancement upon the addition of phosphotyrosine is illustrated in Fig. 1. As shown by the excitation and emission spectrums in Fig. 1a, 100 μM phosphotyrosine enhanced the fluorescence of 100 μM Tb^{3+} from a level of no detectable fluorescence to a readily observable one. Concomitant with phosphotyrosine enhancement of Tb^{3+} fluorescence at 488 and 540 nm was the appearance of a new peak in the excitation spectrum at 275 nm, a region of known strong tyrosine ultraviolet absorption (6). Also shown in the spectrums are peaks that are present regardless of whether conditions necessary for Tb^{3+} fluorescence enhancement had been achieved. It is likely that the peaks in 550 and 244 nm regions are artifacts of Rayleigh light scattering by sample molecules that occur when emission spectra are recorded for 275 nm excitation light and when excitation spectrum are observed at the emission wavelength of 488 nm. The peak in the 600 nm region is likely a Rayleigh scatter peak of the fluorescence emitted from aromatic phosphotyrosine sidechain in the 302 nm region when exited by 275 nm light (6). It is seen both in the presence and absence of Tb^{3+} and thus is not a result of Tb^{3+} fluorescence enhancement. Although for convenience, Tb^{3+} fluorescence enhancement was recorded at 488 nm to avoid possible contributions from scatter peaks, observations at the more intense 540 nm peak can be made if corrected for background light-scatter contributions. As indicated in Fig. 1b, the intensity of Tb^{3+} fluorescence enhancement varied directly as function of phosphotyrosine concentration. Plots of log

fluorescence intensity versus log phosphotyrosine concentration yielded straight line graphs from 1-50 μM and 0.5-100 μM phosphotyrosine for 100 μM and 400 μM Tb^{3+}, respectively. Furthermore, while the rates of change in fluorescence at both Tb^{3+} concentrations were essentially the same at the two Tb^{3+} levels, the overall magnitude of fluorescence/phosphotyrosine in the regions of linear response was 2-fold higher with 400 μM Tb^{3+} than with 100 μM Tb^{3+}.

Stoichiometry and strength of binding of Tb^{3+} to phosphotyrosine. Tb^{3+} titration of phosphotyrosine, using the fluorescence enhancement at 488 nm to monitor the interaction, was used to determine the number of binding sites (n) and association constant (K_a) for the complex. For the equilibrium between Tb^{3+} and phosphotyrosine (PT) in which Tb^{3+} + PT = Tb^{3+}:PT and K_a = [Tb^{3+}:PT]/[Tb^{3+}][PT], there is the linear equation of the form:

$$\frac{[Tb^{3+}]}{[\text{bound-PT}]} = \frac{1}{nK[\text{free-PT}]} + \frac{1}{n}$$

A plot of [Tb^{3+}]/[bound-PT] versus 1/[free-PT] should give a straight line where the y intercept equals 1/n and where K_a equals the y intercept/slope (8,9). Figure 2 depicts the results of the Tb^{3+} titration

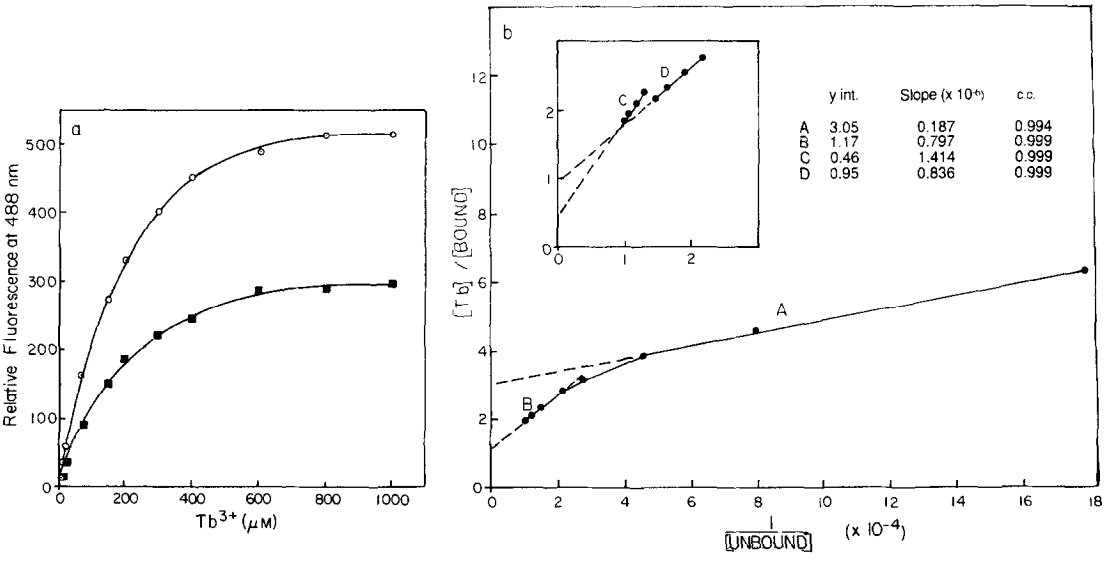

Figure 2. Titration of phosphotyrosine by Tb^{3+}. (a) Tb^{3+} fluorescence enhancement by 50 μM (▪) and 100 μM (○) phosphotyrosine in the presence of 1 to 1000 μM Tb^{3+}. Conditions for fluorescence determinations are given in Materials and Methods. Each point is the mean of 3 or more independent determinations, standard errors about the means were smaller than the graphical space occupied by the data points. (b) Plot of [Tb^{3+}]/[bound-PT] versus 1/[free-PT] (see text) for phosphotyrosine binding to Tb^{3+}. Data are replotted from the 100 μM phosphotyrosine data in Figure 2a. The binding parameters calculated for the lines in 2b are: A, Ka = 1.63 x 10^5 M^{-1} and n = 0.33; B, Ka = 1.47 x 10^4 M^{-1} and n = 0.85; C, Ka = 3.25 x 10^3 M^{-1} and n = 2.17; D, Ka = 1.14 x 10^4 M^{-1} and n = 1.05.

curve and a replotting of the data for the purpose of determining n and K_a. As shown in Fig. 2a, the fluorimetric titration of phosphotyrosine shows the fluorescence dependence on [Tb^{3+}] followed a hyperbolic curve. A further analysis of the fluorescence binding curve, Fig. 2b, indicated the number of ligands complexed with Tb^{3+} varied during the titration. Over the [Tb^{3+}] ranges of 1-150 μM (line B) and 200-600 μM (line A) the number phosphotyrosines bound/Tb^{3+} went from 0.85 to 0.33. Furthermore, a more detailed analysis of the 1-150 μM Tb^{3+} concentration range indicated (see Fig. 2b insert) that the number of phosphotyrosines bound/Tb^{3+} of the 1-50 μM Tb^{3+} range (line C) was 2.17 and over the 70-150 μM Tb^{3+} range (line D) was 1.05. Thus the ratio of phosphotyrosine/Tb^{3+} during a Tb^{3+} titration of 100 μM phosphotyrosine would appear to change from 2/1 for 1-50 μM Tb^{3+} to 1/1 for 70-120 μM Tb^{3+} and reach an endpoint of 1/3 at high Tb^{3+} concentrations. This likely reflects changing stereochemistry of the complex resulting from the interactions of increasing numbers of the polycationic Tb^{3+} with polyanionic phosphotyrosine, i.e., pK_1 < 2 (phosphate) pK_2 2.4 (COOH) and pK_3 5.8 (phosphate) (6). The corresponding apparent K_a values for the lines in Fig. 2b range from 3.25×10^3 M^{-1} to 1.63×10^5 M^{-1} and are comparable to values similarly determined for the binding of Tb^{3+} with nucleic acids (8-10).

Comparison of phosphotyrosine with other biochemical compounds for the ability to enhance Tb^{3+} fluorescence. It was of interest to determine the degree to which the Tb^{3+} fluorescence enhancing properties of phosphotyrosine may be unique. Shown in Table I is a survey the Tb^{3+} fluorescence enhancing properties of other amino acids and phosphorylated biochemical compounds. Among unphosphorylated amino acids, including tyrosine, as well as for phosphoserine and phosphothreonine, there was no detectable Tb^{3+} fluorescence enhancement. Interestingly, tyrosine-O-sulfate, a widespread post-translational form of tyrosine in proteins (11), yielded neither detectable Tb^{3+} fluorescence enhancement when excited at 275 nm nor an excitation peak at 275 nm when Tb^{3+} was added. Except for GMP, there was also little or no detectable enhancement of Tb^{3+} fluorescence by various other phosphorylated nucleosides, sugars or choline. The ability of GMP, and other nucleotides containing guanine, to enhance Tb^{3+} fluorescence has been reported (12,2) and was expected to show enhancement under the present experimental conditions.

DISCUSSION

As shown here, readily detectable Tb^{3+} fluorescence enhancement results when Tb^{3+} is in the presence of phosphotyrosine. The fluorescence wavelengths are characteristic for Tb^{3+} and would appear to result from an energy transfer of ultraviolet light absorbed by the tyrosine aromatic sidechain to Tb^{3+}. The

Table I: Tb^{3+} fluorescence enhancing properties of other amino acids and various other compounds

Compounds (100 μM)	Tb^{3+} Fluorescence[a] (rel. intensity)
Amino acids:	
phosphotyrosine	254 ± 2[b]
phosphoserine	< 1
phosphothreonine	< 1
tyrosine-O-sulfate	< 1
tyrosine	< 1
phenylalanine	< 1
tryptophan	< 1
histidine	< 1
other common amino acids	< 1
Other compounds:	
GMP	74 ± 1
other common nucleotides (CMP, UMP, TMP, AMP)	< 2
deoxyribose phosphate	< 1
phosphoryl choline	< 1
glucosamine-6-phosphate	8 ± 1
galactosamine-1-phosphate	< 1
NAD, NADP	< 6

[a] Tb^{3+} concentration was 400 μM, other conditions for fluorescence measurement were as described in Materials and Methods.

[b] Values were determined from 3 or more independent measurements and are reported as means ± S.E.M.

presence of the phosphate on phosphotyrosine is essential for efficient fluorescence production and likely reflects a requirement for holding Tb^{3+} in close proximity, through electrostatic interaction, to allow efficient energy transfer.

The emission from Tb^{3+} may have a number of uses in the study phosphotyrosine. Its selective detection of phosphotyrosine may allow its use in the analysis of protein phosphotyrosine content. In addition, it may prove useful in monitoring protein conformational and structural changes during the interaction of protein phosphotyrosine with other macromolecules or small cations. Further studies at the level of Tb^{3+} fluoresence enhancement by polypeptide forms of phosphotyrosines will aid in the assessment of its usefulness at the polymer level.

REFERENCES

1. Brittain, H.G., Richardson, F.S., and Martin, R.B. (1976) J. Amer. Chem. Soc. 98, 8255-8260.
2. Ringer, D.P., Burchett, S., and Kizer, D.E. (1976) Biochemistry 17, 4818-4825.

3. Ringer, D.P., Howell, B.A., and Kizer, D.E. (1980) Anal. Biochem. 103, 337-342.
4. Ringer, D.P., Etheredge, J.L., and Kizer, D.E. (1985) J. Inorgan. Biochem. 24, 137-145.
5. Hunter, T., and Cooper, J.A. (1985) Ann. Rev. Biochem. 54, 897-930.
6. Cooper, J.A., Sefton, B.M., and Hunter, T. (1983) Methods in Enzymology 99F, 387-402.
7. Glenney, J.R., Zokas, L., and Kamps, M.P. (1988) J. Immunol. Meth. 109, 277-285.
8. Topal, M.D., and Fresco, J.R. (1980) Biochemistry 19, 5531-5537.
9. Arquilla, M., Thompson, L.M., Pearlman, L.M., and Simpkins, H. (1983) Cancer Res. 43, 1211-1216.
10. Gross, D.S., and Simpkins, H. (1981) J. Biol. Chem. 256, 9593-9598.
11. Huttner, W.B. (1982) Nature (Lond.) 299, 273-276.
12. Formoso, C. (1973) Biochem. Biophys. Res. Commun. 53, 1084-1087.