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HIGH AFFINITY CALCIUM BINDING SITES ON ERYTHROCYTE MEMBRANE PROTEINS

USE OF LANTHANIDES AS FLUORESCENT PROBES

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SUMMARY

1. We have explored the utility of lanthanide cations as probes for calcium binding sites in biomembranes using the erythrocyte ghost as a model system.

2. Interaction of terbium with erythrocyte ghosts produces appearance of a new excitation peak (295 nm), enhancement of terbium fluorescence and quenching of tryptophan fluorescence.

3. The excitation characteristics of bound terbium and the enhancement of its fluorescence by intramolecular energy transfer suggest that tyrosyl hydroxyls are ligands for this cation.

4. Excess calcium can displace terbium from its membrane binding sites. Magnesium is a less effective competitor.

5. The use of rare earths as proximity probes is also discussed.

INTRODUCTION

Calcium actively participates in many biomembrane phenomena. For example, intercellular communication [1], cellular adhesion [2], neurotransmitter release [3], and potassium transport [4] all require calcium. Unfortunately, calcium lacks the chemical and physical properties which allow detailed experimental study of the mechanism by which this divalent cation participates in divers functions of biomembranes.

It has been suggested that lanthanides (rare earth metals) might provide suitable probes for calcium binding sites [5, 6]. Not only do these metals provide a graduated series of atomic radii approximately the same as that of calcium but they are also fluorescent and paramagnetic. Luk [7] has used the fluorescent properties of terbium to probe the metal binding sites of transferrin. Reports have appeared in the literature employing the paramagnetic properties in studies of the

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calcium binding sites of proteins [8] and sidedness of lipid bilayers [9]. In this report, we demonstrate the utility of lanthanide fluorescence in exploring the calcium binding sites of human erythrocytes.

THEORY

Because of the nature of the data to be presented, we must provide an introductory discussion of the fluorescent properties of rare earths. More detailed information is available in the excellent review by Sinha [10]. Certain rare earth chelates when irradiated with light absorbed by the organic ligand emit light with a spectra energy distribution characteristic of the rare earth ion. In recent years, this process has been extensively investigated due to the possible laser action of some of these chelates. The mechanism for this intramolecular energy transfer from ligand to central atom has been delineated by Crosby and associates [11, 12]. It involves excitation of the organic ligand to an excited singlet state, followed by nonradiative deactivation to the lowest vibrational level of the excited singlet. The chelate can either drop down to the ground state by nonradiative decay or by a radiative process, (ligand fluorescence), or undergo a nonradiative transition to the triplet energy level of the ligand. From the excited triplet state, the molecule can return to the ground state by collisional quenching processes or molecular phosphorescence. Alternatively, the energy can be transferred to the central atom with resultant ion fluorescence characteristic of the rare earth. Of most of the lanthanide chelates studied, those with samarium, europium, terbium or dysprosium as the central ion show strong ion fluorescence via intramolecular energy transfer. This is probably the result of sufficient overlap of the triplet energy level of the donor ligands and the resonance energy level of the rare earth metal. Possible biological ligands that have triplet energy levels near the absorbance regions of the rare earths include tyrosine and tryptophan with phosphorescence emission maximum at 395 nm and 490 nm, respectively [13].

EXPERIMENTAL

Materials

Rare earths are supplied by Alpha Chemicals (Ventron) as the chloride salts. All other chemicals are reagent grade. Nalgene plasticware is used throughout the experiments to the exclusion of all glassware, to minimize contamination by extraneous calcium.

Procedures

We prepare erythrocyte ghosts from fresh heparinized human O^+ blood by the method of Dodge et al. [14]. The ghosts are lysed according to Dodge et al. and then washed with 1 mM EDTA buffered in 0.02 M Tris–HCl plus 0.13 M NaCl (pH 7.1), followed by four buffer washes to remove the EDTA. Sodium dodecylsulfate-polyacrylamide gel electrophoresis demonstrates that "spectrin" is not eluted by the washing step; this is consistent with the high ionic strength involved [15].

For fluorescent titrations we employ an Hitachi/Perkin Elmer MPF3 spectrofluorometer yielding corrected emission and excitation spectra. In a typical experiment, ghosts ($< 50\ \mu g/ml$ of protein) in 0.02 M Tris–HCl plus 0.13 M NaCl

at pH 7.4 are titrated with lanthanides in water so that the final volume change for the entire titration curve is less than 5 %. At the protein concentrations employed, 'inner filter' effects are not significant.

Protein is determined as in ref. 15.

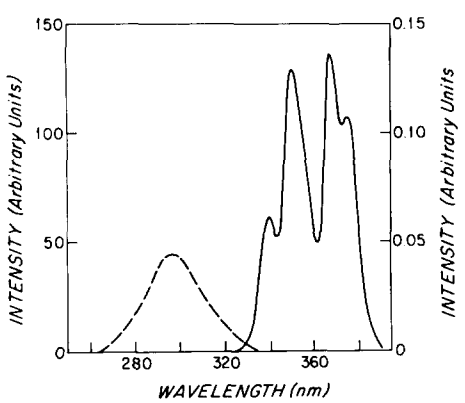
RESULTS

Figs 1 and 2 depict the excitation and emission spectra, respectively, for $TbCl_3$ in water. The excitation spectrum is characterized by several absorbance bands in the 340–400 nm range. Excitation by light in this energy range yields three degenerate emission bands at 490 nm, 545 nm and 590 nm (Fig. 2).

When one equilibrates erythrocytes with low concentrations (10^{-6}–10^{-5} M) of Tb^{3+}, two important spectral changes appear: (1) the excitation maximum shifts from around 350 nm to 295 nm, and (2) the fluorescence is greatly enhanced. However, the fluorescence emission spectrum remains unchanged. The increase in fluorescence is in the order of 10^3 (Fig. 2) with excitation at 295 nm but there is no detectable enhancement in fluorescence with excitation at 352 nm.

Fig. 3, a typical titration of erythrocyte ghosts with Tb^{3+}, illustrates several important facts. First, fluorescence enhancement reaches a limit with increasing Tb^{3+} concentration; it thus represents binding rather than random collisional processes. Furthermore, enhancement of Tb^{3+} fluorescence is accompanied by quenching of tryptophan emission. Finally, the binding is very tight and cannot be blocked by high monovalent salt concentrations even at low Tb^{3+} concentrations. This suggests that Tb^{3+} is actually chelated (and not simply complexed) by the erythrocyte membranes.

Fig. 3 also demonstrates an experiment where the erythrocyte ghosts were exposed to buffered 0.1 % sodium dodecylsulfate. This produces a large decrease in



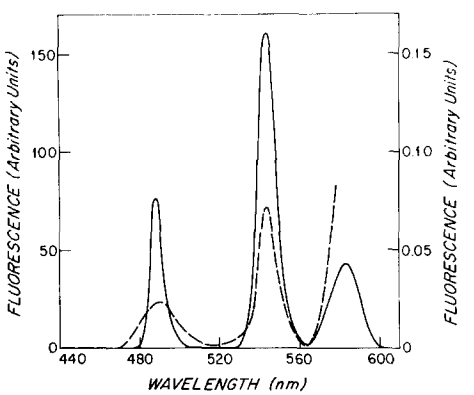


Fig. 1. Corrected fluorescence excitation spectra for Tb^{3+}. (—): 0.02 M $TbCl_3$ in water. (- - -): 10^{-5} M Tb^{3+} plus 37 μg/ml ghost protein in 0.02 M Tris–HCl, 0.13 M NaCl (pH 7.1). The emission wavelength is 489 nm. Similar results are obtained at 545 nm. Excitation slit 8 nm; emission slit 10 nm.

Fig. 2. Corrected fluorescence emission spectra for Tb^{3+}. Conditions and symbols as in Fig. 1. Excitation wavelength is 352 nm for free Tb^{3+} and 295 nm for Tb^{3+} in the presence of ghosts. Light scattering background has been subtracted.

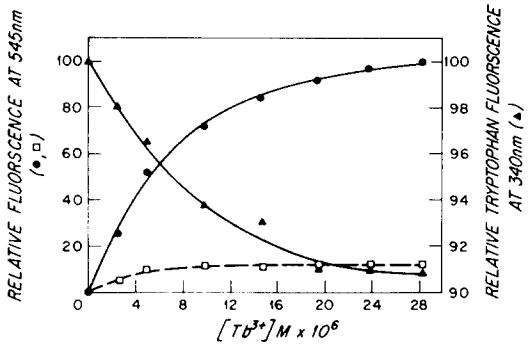


Fig. 3. Titration of ghosts with Tb^{3+}. Excitation at 295 nm and emission at 545 nm. (● — ●); 44 μg/ml ghost protein in 0.02 M Tris–HCl, 0.13 M NaCl (pH 7.1); (□ — □): 0.1 % sodium dodecylsulfate, 0.02 M Tris–HCl, 0.13 M NaCl (pH 7.2); (▲ — ▲) Excitation at 286 nm and emission at 340 nm (tryptophan) at 44 μg/ml ghost protein in 0.02 M Tris–HCl, 0.13 M NaCl (pH 7.1).

Tb^{3+} fluorescence, i.e. Tb^{3+} binding, indicating the importance of an intact membrane in the binding process. The decrease is probably not entirely due to competition between the sulfate head of the detergent and the membrane binding sites because of the high ionic strength and the lack of chelation ability of the detergent below its critical micelle concentration (0.23 %).

Evidence that terbium is binding at the calcium binding sites on the erythrocyte membrane comes from the ability of calcium to displace Tb^{3+}. This competition is illustrated in Fig. 4, showing plots of the reciprocal of the fluorescence versus $1/[Tb^{3+}]$ at different calcium levels. The lines are linear at high $[Tb^{3+}]$ but become concave upward at lower concentrations of Tb^{3+} indicating more than one type of binding sites. Such nonlinear double reciprocal plots have been found in many enzyme systems and is indicative of multiple binding sites [17, 18]. In the present context, it is important

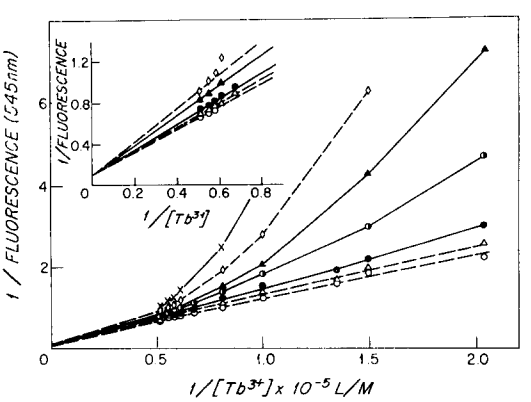


Fig. 4. Competitive inhibition of Tb^{3+} binding to ghosts by Ca^{2+}. Ghost protein concentration 40 μg/ml. The ordinate is the reciprocal of fluorescence (arbitrary units) per mg protein. Calcium concentration: (○ — ○) 0.0 M Ca^{2+}; (△ — △) $2 \cdot 10^{-3}$ M; (● — ●) $4 \cdot 10^{-3}$ M; (◑ — ◑) $5 \cdot 10^{-3}$ M; (▲ — ▲) $6 \cdot 10^{-3}$ M; (▽ — ▽) $8 \cdot 10^{-3}$ M; (× — ×) $9 \cdot 10^{-3}$ M. The insert is a 5-fold magnification of the high $[Tb^{3+}]$ region. Excitation at wavelength 295 nm and emission at wavelength 545 nm.

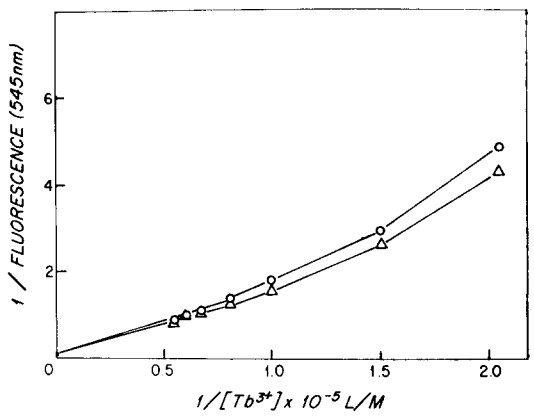


Fig. 5. A comparison of competitive inhibition of Tb^{3+} binding to ghosts by Ca^{2+} and Mg^{2+}. Ca^{2+} (○—○) and Mg^{2+} (△—△) concentration are $5 \cdot 10^{-3}$ M. Excitation at wavelength 295 nm. Ghost protein concentration 40 μg/ml. The ordinate is the reciprocal of fluorescence (arbitrary units) per mg protein.

to note that all curves extrapolate to the same intersection point on the ordinate suggesting competitive inhibition of Tb^{3+} binding by calcium.

At low terbium concentrations, all terbium binding as evidenced by fluorescence is inhibited by calcium. At higher concentrations of Tb^{3+} ($5 \cdot 10^{-5}$ M), there is still some residual fluorescence even at calcium concentrations as high as 0.01 M. Higher calcium concentrations cannot be studied due to aggregation of the ghosts. However, this information supports the evidence from the double reciprocal plots that there are at least two sites for Tb^{3+} and calcium binding. The high concentrations of calcium required to replace Tb^{3+} are not surprising when one compares the stability constants of rare earth chelates with calcium chelates. For example, the stability constant of the EDTA chelate of Eu^{3+} is $10^{17.35}$ compared to $10^{10.6}$ for the EDTA–Ca^{2+} chelate [16].

We have also titrated the Tb^{3+}–ghost complexes with Mg^{2+}. Similar nonlinear double reciprocals plots are obtained as with calcium. The Mg^{2+} cation competes less effectively (Fig. 5).

DISCUSSION

Our data suggest that tyrosine is a ligand of at least one class of binding sites for calcium in erythrocyte ghosts. Our reasoning can be summarized as follows.

(1) The excitation spectrum of bound Tb^{3+} shows no detectable maximum at the excitation peak of free Tb^{3+}, nor at the emission maxima of tyrosine ($\approx$ 310 nm). or tryptophan ($\approx$ 340 nm). The enhanced fluorescence of bound Tb^{3+} can thus not be attributed to resonance energy transfer from tyrosine or tryptophan, but rather represents the properties of complexes of Tb^{3+} with aromatic residues in erythrocyte ghost proteins.

Since excitation of Tb^{3+} fluorescence cannot be ascribed to energy transfer from tryptophan, quenching of tryptophan fluorescence is most reasonably attri-

buted to decreased energy transfer from tyrosine to tryptophan. This must arise at least in part from the shifted emission maximum of the tyrosine–Tb^{3+} complex, but we cannot exclude some paramagnetic deactivation of excited tryptophan molecules.

(2) Tryptophan is dubious ligand because the indole ring does not constitute a "hard base", such as an oxygen donor, necessary to complex calcium or rare earths [19, 20]. Moreover, the spectral overlap between the excited triplet level of tryptophan and the level of Tb^{3+} is negligible whereas that with tyrosine is considerable. We further reason that the excitation band at 295 nm represents the excitation of ionic tyrosine–Tb^{3+} complexes rather than a tryptophan contribution. The wavelength position is correct for ionized tyrosine and, if the 295 band represented a tryptophan transition, one would also expect excitation at lower wavelengths due to the tyrosine–tryptophan energy transfer characteristic of these membranes. Finally, our conclusions are in accord with the work of Luk [7] who has shown that Tb^{3+} can be chelated by tyrosine with a resulting enhancement of ion fluorescence.

(3) The competition binding experiments with calcium indicate that the sites being titrated by Tb^{3+} are the calcium complexing sites.

The lack of substantial discrimination between calcium and magnesium can be explained by at least three possibilities. (1) The membrane sites monitored by Tb^{3+} fluorescence do not exhibit a large preference for Ca^{2+} over Mg^{2+}. (2) Because the stability constant for Tb^{3+} binding is so high in comparison with those for Ca^{2+} and Mg^{2+}, even significant differences between the latter equilibrium constants cannot be distinguished readily. (3) Finally, it is possible that the ability of certain biological systems to differentiate between calcium and magnesium is not dependent upon equilibrium considerations, e.g. stability constants such as measured here. The specificity may, however, reside in kinetic terms. This has been suggested to be the case for certain ATPases that can use either Ca^{2+} or Mg^{2+}. The stability constants for Ca^{2+}–ATP and Mg^{2+}–ATP are about the same but the exchange rates for the complexes with ATP are much different [24].

Earlier work concerning the calcium binding sites of erythrocytes offer little information concerning divalent cation preference. The experiments of Long and Mouat [22], although performed at low ionic strengths, indicate little divalent cation binding specificity by erythrocyte membranes. What is required to investigate the origin of this cation preference in situations where the stability constants are not much different is a functional assay system such as an enzyme. One example which we are currently investigating is the (Na^+–K^+)-ATPase of erythrocytes which is activated by Mg^{2+} and inhibited by Ca^{2+} [25].

Previous work on the calcium binding sites of biomembranes including erythrocyte membranes has been limited to indirect approaches for the reasons stated earlier in the introduction. Seaman et al. [21] utilizing electrophoretic mobility as a measure of calcium binding and the effect of sialidases as an index of sialic acid participations, suggest that these sugars constitute major calcium ligands. However, as these authors rightly point out, such electrophoretic studies give little direct insight into calcium binding. They also report a high affinity binding site for calcium that is apparently unaffected by neuraminidase.

Long and Mouat [22] using ^{45}Ca in binding experiments also propose that sialic acids complexed calcium. However, their experiments are difficult to interpret because they were performed at ionic strengths much lower than physiological.

Their own inhibition studies with monovalent and other divalent cations indicated a substantial decrease in calcium binding with increasing ionic strengths. They also provide evidence for a high affinity binding site that is resistant to neuraminidase.

Finally, Harrison and Long [23] showed that extraction of erythrocytes with chloroform methanol does not affect the calcium binding. They suggest that phospholipids are not involved.

We suggest that the sites monitored by Tb^{3+} fluorescence are the high affinity binding sites referred to by Seaman et al. [21] and Long and Mouat [22]. We are currently in the process of investigating this possibility utilizing the paramagnetic properties of the rare earths.

Our results introduce a novel, useful approach to the identification of alkaline earth ligands in biomembranes; they also suggest another technique for exploring the nature of these binding sites, namely paramagnetic quenching of protein fluorescence. This mechanism of quenching has been proposed by one of us [26] for studies of proximity relationships because it occurs only when the separation between fluorophore and quencher does not exceed 6–7 Å. The utility of this approach has been recently demonstrated using nitroxide stearates interacting with the hydrophobic binding sites of bovine serum albumin [27]. We are exploring this approach using paramagnetic lanthanide cations.

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