TERBIUM AS LUMINESCENT PROBE OF CALMODULIN CALCIUM-BINDING SITES

Domains I and II contain the high-affinity sites

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1. Introduction

Terbium has been shown to be a useful tool in the study of protein calcium binding sites [1]. Due to its ionic radius, its coordination number between 6 and 8 and its strong propensity for oxygen donor groups, this trivalent ion can replace calcium in many biological systems [2]. In addition, terbium has the convenient property of being highly luminescent when it binds to a protein close to an aromatic residue, as a result of a dipole–quadrupole energy-transfer process. By contrast the free ion is only poorly luminescent.

Calmodulin, the ubiquitous and multifunctional calcium-dependent regulator [3–5]exhibits 4 calcium binding sites among which sites III and IV contain one tyrosine residue each (tyrosine 99 in site III and tyrosine 138 in site IV [6]). No other tyrosyl residue is present in the molecule. Upon calcium binding, calmodulin undergoes a conformational transition which is necessary for its biological activity [7–9].

Here, calmodulin conformational changes were induced by Ca^{2+} and Tb^{3+} and studied by monitoring tyrosine fluorescence, since calmodulin contains no tryptophan residues. Two calcium binding sites were shown to be involved in tyrosine fluorescence changes. A study of terbium emission indicates these high affinity sites to be sites I and II, in contrast to troponin C where the high affinity sites are sites III and IV.

2. Materials and methods

All chemicals were the purest commercially available. Ultra pure water (MilliQ instrument from Millipore Corp.) and acid-washed plastic ware were used throughout to minimize calcium contamination. $TbCl_3$ was purchased from Koch-Light. Calmodulin was prepared from ram testis as in [10]. The protein was shown to be indistinguishable from calmodulin isolated from other mammalian organs. In particular, it exhibits the same amino acid composition and tryptic peptide maps as the homologous protein from bovine brain [10].

Calmodulin concentrations were determined by ultraviolet spectroscopy using $\epsilon_M = 3300\ M^{-1} . cm^{-1}$ [8,10]. Correction for scattered light was made as in [11]. Calcium was measured by atomic absorption spectrophotometry using a Varian apparatus Model 1150. Calmodulin was freed of calcium by trichloroacetic acid precipitation (J. Haiech, C. B. Klee, J. G. D., in preparation). Residual calcium was <0.04 mol/mol protein. Reagents were passed through a Chelex-100 column and stored in plastic ware.

2.1. *Fluorescence measurements*

Fluorescence spectra were recorded on an absolute spectrofluorimeter (Fica 55) with an excitation wavelength at 275 ± 2.5 nm. Quantum yields ϕ were determined as in [12], using free tyrosine as reference ($\phi = 0.14$) [13]. Quantum yields were corrected to account for the screening effect of scattered light [14]. Lifetimes (ζ) were measured by the single photoelectron technique [15]. All experiments were done at 22°C.

2.2. *Fluorimetric titrations*

Titration of metal-free calmodulin by Ca^{2+} or Tb^{3+} was performed by adding aliquots of $CaCl_2$ or $TbCl_3$ stock solutions. $TbCl_3$ solutions were freshly

Abbreviation: MOPS, morpholinopropane sulfonic acid

prepared and kept at 4°C to avoid precipitation. Tb^{3+} titration of metal-free calmodulin was carried out in 100 mM Tris buffer (pH 6.9) or in 20 mM MOPS buffer (pH 6.5). pH was maintained constant throughout the experiment. After each $TbCl_3$ addition, the absence of protein precipitation was checked.

The stoichiometries of the complexes were determined from experiments done at high protein concentrations (⩾10-fold higher than the dissociation constant). Under these conditions added Ca^{2+} or Tb^{3+} was assumed to be protein-bound.

3. Results and discussion

Ca^{2+} binding to calmodulin induces a large increase of the tyrosine fluorescence quantum yield from 0.027 for the calcium free protein to 0.08 at saturating calcium. These results are in agreement with reports of increase in the fluorescence intensity around 303 nm [16,17].

Detailed analysis of this fluorescence variation, including measurements of decay time and accessibility to ionic quenchers, suggests that the quantum yield increase is mainly due to a 'dequenching' of tyrosine 138 (M.-C. K., J. G. D., D. G., in preparation). As shown in fig.1, the total emission change

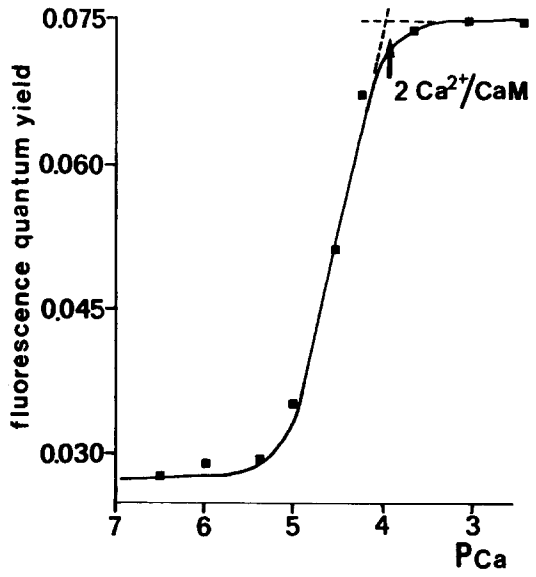

Fig.1. Titration of metal-free calmodulin by Ca^{2+}. Conformational changes induced by successive additions of Ca^{2+} to 4.5×10^{-5} M calmodulin in 100 mM Tris buffer (pH 7.6) were followed as in section 2. The fluorescence quantum yield was plotted against pCa (= −log $[Ca^{2+}]$).

occurred upon binding of 2 mol Ca^{2+}/mol calmodulin, in agreement with circular dichroic and ultraviolet difference spectroscopic studies [8]. NMR studies also point to a first transition upon binding of 2 mol Ca^{2+}/mol calmodulin [18].

Tb^{3+} binding to calmodulin was analyzed by monitoring tyrosine fluorescence, and terbium emission at 545 nm for the same 275 nm excitation wavelength. As shown above for Ca^{2+}, binding of 2 mol Tb^{3+}/mol calmodulin induces an increase in the tyrosine fluorescence quantum yield, that remains however smaller than that induced by Ca^{2+} binding. Such differences between Ca^{2+} and lanthanides may occur as a result of differences in the induced 3-dimensional structures [19]. Up to 2 Tb^{3+} bound/mol protein, Tb^{3+} luminescence remains very weak (fig.2).

Binding of additional Tb^{3+} produces a large decrease in the tyrosine fluorescence quantum yield,

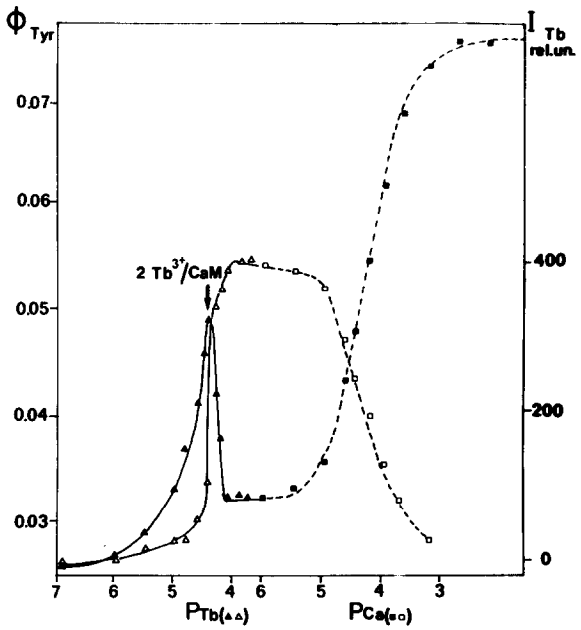

Fig.2. Terbium binding to calmodulin. Solid lines indicate the fluorescence quantum yield (ϕ Tyr) of calmodulin (▲) and the concomitant variation of terbium luminescence at 545 nm: I_{Tb} (△) as a function of added Tb^{3+} (pTb = −log $[Tb^{3+}]$). Ca^{2+}-induced Tb^{3+} removal is represented by dashed lines. (■) stands for tyrosine quantum yield and (□) for terbium luminescence. Calmodulin concentration was 2.2×10^{-5} M in 100 mM Tris buffer (pH 6.9). The same effects were observed by using a 20 mM MOPS buffer (pH 6.5). When Tb^{3+} was $>3 \times 10^{-4}$ M, a Tb^{3+}-induced precipitation of calmodulin occurred that prohibited the spectroscopic study.

which is accompanied by a 400-fold enhancement of terbium emission. All Tb^{3+} effects are abolished upon subsequent addition of Ca^{2+}, indicating that both cations compete for the same sites. The terbium and calcium binding studies described herein provide information about the metal binding sites that are involved in the conformational changes of calmodulin.

The observed tyrosine fluorescence increase is due to the binding of 2 Tb^{3+} to sites which probably lack tyrosine residues, since no enhancement of terbium emission occurred. This suggests that the high affinity binding sites are sites I and II. Nevertheless, binding to sites III or IV, although much less likely, cannot be totally excluded if the spatial configuration of these sites happens to be quite unfavorable to the energy transfer between Tb^{2+} and the aromatic ring. Further terbium binding occurs unambiguously at sites III and/or IV since a large terbium luminescence can then be observed. The excitation spectrum shows a maximum around 280 nm (not shown) which clearly indicates that tyrosine residues are involved in the transfer process. Moreover, Tb^{3+} binding to these sites strongly quenches tyrosine fluorescence. Such an effect, observed for various proteins [1,20], was generally considered as the consequence of the energy transfer. However, energy transfer involves the triplet state of the aromatic chromophore [21] and cannot account for most of the fluorescence quenching which indeed originates from the first singlet excited state. Therefore, the observed tyrosine quenching is likely to be essentially due to the singlet quenching property of Tb^{3+} toward the phenol ring, similar to the one reported for indole ring quenching [22]. Tb^{3+} is permanently maintained close to the tyrosine residue by the geometry of the metal binding site; this explains the efficient quenching even at low Tb^{3+} concentrations (5×10^{-5} M). Also the lifetime remains constant (~2 ns) when tyrosine fluorescence quenching occurs, indicating that quenching is not controlled by diffusion. Taking the Ca^{2+} and Tb^{3+} binding experimental results together, the conformational changes revealed by fluorescence changes appear to occur upon binding of 2 mol ion/mol protein at high affinity sites I and II, that are devoid of tyrosyl residues. In contrast, NMR studies [18] pointed to sites III and IV as being the high affinity sites. However, resonances of both tyrosines could well be affected by Ca^{2+} binding to sites I and II as a result of a global conformational change of the molecule. Sensitized terbium luminescence, which can only originate from close spatial relationships between the lanthanide and phenol rings, is a more clear cut probe for the sequential ion binding, firstly to tyrosine-free sites then to tyrosine containing sites. In spite of a close cladistic relationship between troponin C and calmodulin [6,23], these 2 intracellular Ca^{2+} binding proteins exhibit quite different ion binding properties. Troponin C high affinity sites are located in domains III and IV [19,24,25], whereas such sites are present in calmodulin domains I and II.

However, calmodulin was shown to replace troponin C in the Ca^{2+}-activation of actomyosin ATPase [26], and troponin C is able to replace calmodulin in the activation of glycogen phosphorylase *b* kinase [27]. In all cases, since Mg^{2+} is present at mM levels and saturates sites III and IV of troponin C, sites I and II are in both cases involved in the first steps of the Ca^{2+}-dependent activation of biological processes.

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