TERBIUM BINDING TO OCTOPUS CALMODULIN PROVIDES THE COMPLETE SEQUENCE OF ION BINDING

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1. Introduction

Calmodulin, the ubiquitous and multifunctional calcium-dependent regulator [1,2], exhibits 4 calcium-binding sites. For each of these sites, Ca^{2+} compete with Mg^{2+} and K^+ (J. Haiech, C. B. Klee, J. G. D., in preparation). Binding of Ca^{2+} to calmodulin induces a conformational change which was observed by different techniques, CD and UV difference spectra [3], NMR [4] or tyrosine fluorescence ([5], M. C. K., J. G. D., D. G., in preparation). Ca^{2+}-loaded calmodulin is able to interact with and activate a number of enzymes (reviewed [1,2]). It is still a matter of speculation whether all enzymes are activated by calmodulin when loaded with 4 Ca^{2+} or whether calmodulin can activate some enzymes after binding 2 or 3 Ca^{2+} only.

It is therefore important to determine the sequence of ion binding to the 4 domains recognized in the primary structure of the protein [6]. Homologous calmodulins in spite of a very low rate of evolution [7] exhibit amino acid substitutions that may be useful in this respect. For instance, mammalian calmodulins contain two tyrosyl residues at positions 99 (domain III) and 138 (domain IV) [6]. We have recently shown that 'high affinity' sites are sites I and II, since Tb^{3+} luminescence increases only after binding of 2 Tb^{3+} equivalents. The 2 first Tb^{3+} therefore bind to sites that lack tyrosine, namely domains I and II [5]. Invertebrate calmodulins appear to contain only 1 tyrosyl residue [8,9], located in the *Renilla reniformis* protein in a position homologous to the one of tyrosine 138 of bovine brain calmodulin [9].

We report here the localization of the single tyrosyl residue of octopus calmodulin in the same position, and the use of terbium in completing the following ion binding sequence. After binding to the high affinity domains I and II, Tb^{3+} bind to domain III, and finally to the lower affinity domain IV.

2. Materials and methods

Octopus calmodulin was purified to homogeneity and shown to contain 1.0 mol tyrosine/mol as described elsewhere (J. G. D., M. C. K., C. Ferraz, E. Audemard, M. P. Walsh, submitted). The tyrosine containing tryptic peptide was isolated by HPLC as in [10] and its amino acid composition determined after 24 h hydrolysis in 5.6 N HCl at 110°C.

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. Fluorescence measurements (excitation wavelength 280 ± 2.5 nm) and fluorimetric titrations were performed in 20 mM MOPS buffer (pH 7.1 or 6.9) as in [5]. Calmodulin concentrations were determined by amino acid analysis of aliquots withdrawn from the cuvette at the end of each experiment. Since protein levels were >20 μM, added Ca^{2+} or Tb^{3+} was assumed to be protein-bound, at least for the 3 first sites to be saturated.

Abbreviations: MOPS, morpholinopropane sulfonic acid; Tris, tris(hydroxymethyl) amino methane

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3. Results and discussion

3.1. *The octopus calmodulin single tyrosyl residue is located in domain IV*

Octopus calmodulin exhibits a characteristic UV absorption spectrum (fig.1) with the vibronic structure of phenylalanyl residues at 252.5, 258.5, 264.5 and 268 nm and the tyrosine peak at 278 nm. The absorption coefficient $\epsilon_{mM,\ 278\ nm} = 2.4$ is significantly lower than the one found for mammalian calmodulins ($\epsilon_{mM,\ 277\ nm} = 3.3$) [3,10] which contain 2 tyrosyl residues. Although $\epsilon_{278\ nm}$ appears to be somewhat higher than expected from a single

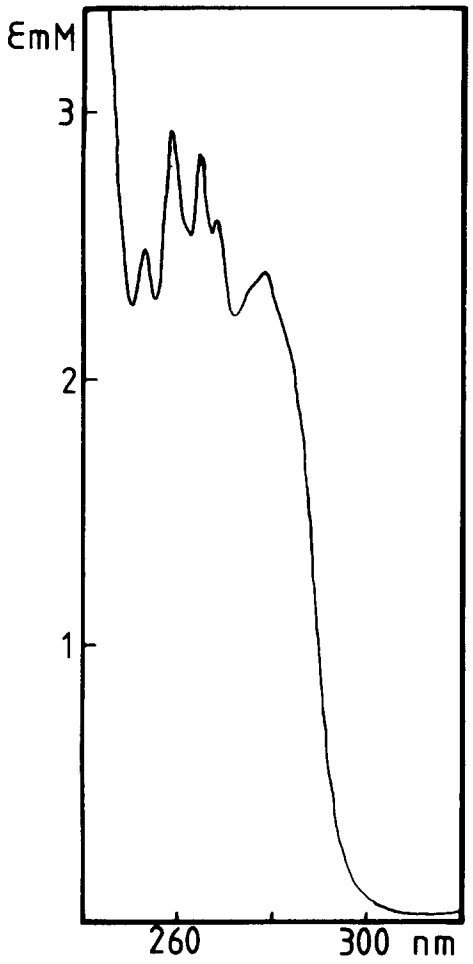

Fig.1. Ultraviolet absorption spectrum of octopus calmodulin. The protein was dissolved in 20 μM $CaCl_2$, 0.27 M NaCl, 1 mM magnesium acetate, 20 mM imidazole buffer (pH 6.5). Extinction coefficient was obtained by amino acid analysis of an aliquot of the sample.

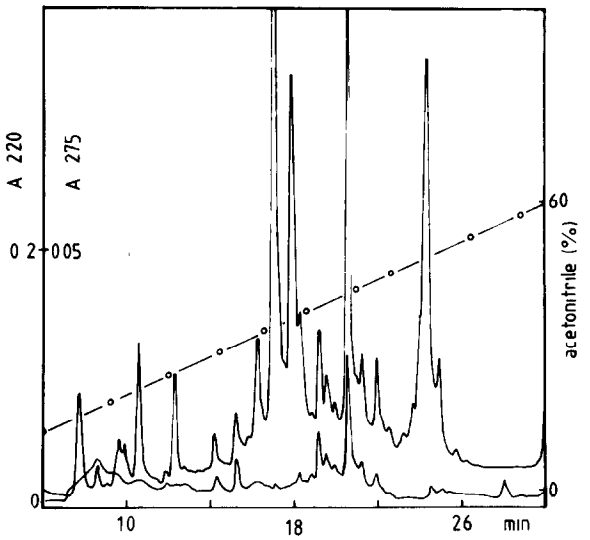

Fig.2. High performance liquid chromatography of an octopus calmodulin tryptic digest (10 nmol) on a μ Bondapak Phenyl column [10]. Upper trace: A_{220} of the eluate, 0.4 absorbance units full scale. Lower trace: A_{275} of the eluate, 0.1 absorbance units full scale. Lower and upper traces were obtained on different and consecutive runs. In the illustrated part of the run (6–30 min), the acetonitrile gradient (o——o) was from 12–60%.

tyrosyl residue, the amino acid analysis (J. G. D., M. C. K., C. Ferraz, E. Audemard, M. P. Walsh, submitted) pointed to 1.0 mol tyrosine/mol.

We have shown [10,11] that the tyrosine-containing tryptic peptides of vertebrate calmodulins could be easily separated and identified by HPLC. Fig.2 shows the octopus calmodulin peptide map which is similar but not identical to those of ram testis, bovine brain [10] and *Xenopus laevis* oocyte [11] calmodulins. A peptide is eluted in the same position (16.2 min) as one tyrosine-containing peptide of mammalian calmodulin, but it fails to absorb UV light at 275 nm. One major peptide only does absorb at 275 nm and is eluted at 20.5 min, very close to the other tyrosine-containing peptide of mammalian calmodulins (20.9 min).

The composition of this peptide peak is shown in table 1 and compared with peptides 91–106 (containing Tyr 99) and 127–148 (containing Tyr 138) of bovine brain calmodulin [6]. The 21–22 residue long octopus calmodulin peptide which contains 4 Glx residues, 1–2 methionines and neither leucine nor arginine, is obviously homologous to peptide 127–148 of mammalian calmodulins. Therefore, the

Table 1
Amino acid composition of tyrosine-containing tryptic peptides from octopus and bovine brain calmodulins[a]

Residue	Octopus calmodulin peptide eluted at 20.5 min		Bovine brain calmodulin	
	Found	Integer	Peptide 127–148	Peptide 91–106
Asx	4.0	4	4	3
Thr	1.0	1	1	0
Ser	1.1	1	0	1
Glx	4.0	4	5	1
Gly	2.0	2	2	2
Ala	1.7	2	2	2
Val	1.9	2	2	1
Met	1.5	1-2	2	0
Ile	1.1	1	1	1
Leu	0.3	0	0	1
Tyr	0.8	1	1	1
Phe	0.8	1	1	1
Lys	0.9	1	1	1
Arg	0	0	0	1
	Total	21–22	22	16

[a] Expressed as residues/mol, after 24 h hydrolysis

Values for bovine brain calmodulin peptides were from [6]

single tyrosyl residue of octopus calmodulin is located in a position homologous to Tyr 138 of the bovine brain protein, in the domain IV of the peptide chain.

3.2. *Ca^{2+} binding to octopus calmodulin*

Upon addition of $CaCl_2$ to metal-free calmodulin, the tyrosine fluorescence quantum yield increased from 0.024–0.048 (fig.3). Addition of Ca^{2+} did not change fluorescence life times (2.5 ± 0.2 ns), pointing to static quenching of domain IV tyrosine in the absence of Ca^{2+} (M. C. K., J. G. D., D. G., in preparation). The first linear part of the titration curve, when extrapolated, intersected the plateau level at 1.5 mol Ca^{2+}/mol calmodulin. The conformational change responsible for the increase in the tyrosine fluorescence quantum yield was 92% complete when 2 mol Ca^{2+}/mol calmodulin were added.

Octopus calmodulin is therefore similar to mammalian calmodulins, in that the conformational change monitored by tyrosine fluorescence is essentially complete after binding of 2 mol Ca^{2+}/mol protein [5], in line with NMR [4], CD and UV difference spectroscopic studies [3].

The only apparent difference between octopus and mammalian calmodulins being the absence of a tyro-

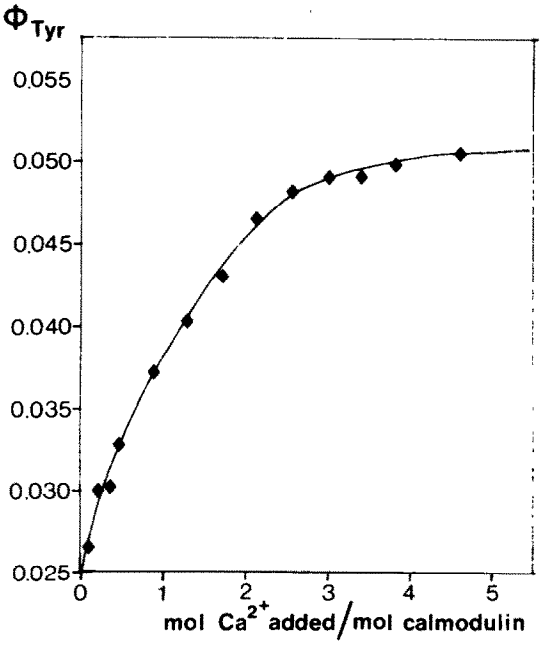

Fig.3. Titration of metal-free calmodulin by Ca^{2+}. Ca^{2+} ($CaCl_2$) were added to 23.3 μM calmodulin in 20 mM MOPS buffer (pH 7.1) and were assumed to be protein-bound. Φ_{Tyr} is the tyrosine fluorescence quantum yield (♦——♦), determined by using free tyrosine as reference (Φ = 0.14) [5].

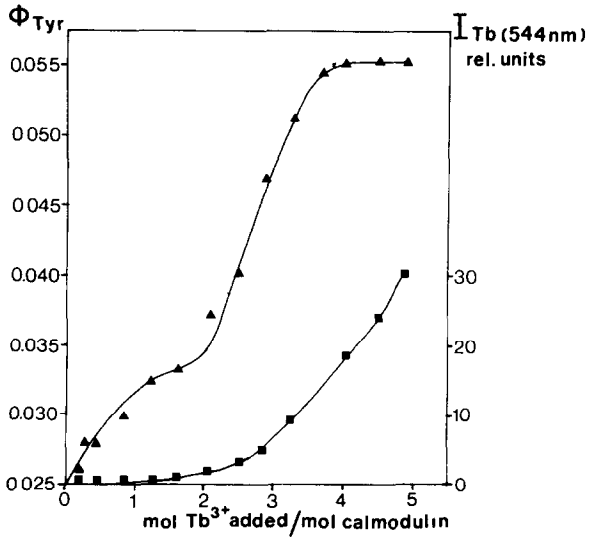

Fig.4. Titration of metal-free calmodulin by Tb^{3+}. Tb^{3+} ($TbCl_3$) were added to 21.7 μM calmodulin in 20 mM MOPS buffer (pH 6.9) and were assumed to be protein bound. Φ_{Tyr} is the tyrosine fluorescence quantum yield (▲——▲), determined as in fig.3. I_{Tb} is the variation of terbium luminescence (■——■) at 544 nm, expressed in relative units, upon excitation at the same wavelength (280 nm).

sine phenol group in domain III, it was possible to determine whether the third ion binds to either domain III or domain IV or both simultaneously, by using Tb^{3+} as a luminescent probe.

3.3. *Terbium binding to octopus calmodulin*

The tyrosine fluorescence quantum yield increased from 0.024–0.055 upon addition of $TbCl_3$ to metal-free calmodulin (fig.4). The increase in fluorescence was biphasic with a first step (up to 0.032) reached after addition of 1 mol Tb^{3+}/mol protein, followed by a larger increase upon further Tb^{3+} addition. In contrast to ram testis calmodulin, which exhibited a marked quenching of tyrosine fluorescence upon addition of more than 2 mol Tb^{3+}/mol [5], no quenching was observed at any step of the Tb^{3+} titration of octopus calmodulin, and the fluorescence plateau, reached for ~3.5 mol Tb^{3+}/mol protein, was comparable to the one obtained with Ca^{2+} (0.055 vs 0.048, respectively). The following conclusions can therefore be drawn:

(i) The Tb^{3+}-induced tyrosine fluorescence quenching observed with mammalian calmodulin involved Tyr 99;

(ii) Mammalian calmodulin site III was saturated immediately after high affinity sites I and II since quenching occurred just after binding of the 2 first Tb^{3+} [5].

These conclusions are supported by the study of Tb^{3+} luminescence upon excitation at 280 nm (fig.4). Tb^{3+} luminescence was negligible up to 2.9 mol Tb^{3+}/mol calmodulin and increased afterwards 17-fold and 30-fold at 4 and 5 mol/mol, respectively. Since Tb^{3+} luminescence is enhanced upon binding of Tb^{3+} close to a phenol group, the third Tb^{3+} binds to domain III which lacks tyrosine, and the fourth to domain IV which contains the single tyrosyl residue of the molecule. Also, the observed luminescence of Tb^{3+}-loaded octopus calmodulin is small when compared with the 400-fold enhancement of luminescence observed with mammalian calmodulin [5], suggesting that domain IV has a significantly lower affinity for Tb^{3+} than domain III.

In conclusion, the sequence of Tb^{3+} binding to calmodulin can be described as follows: Domains I and II, followed by domain III, and finally the lower affinity domain IV are sequentially saturated by the lanthanide, and presumably by Ca^{2+} in vivo since Tb^{3+} and Ca^{2+} compete for the same sites. It is therefore conceivable that, when pCa decreases from 8–5 upon stimulation of the intact cell, different classes of enzymes may be sequentially activated. It is tempting to speculate that the conformational change induced by Ca^{2+}-binding to sites I and II is similar to the one induced by Ca^{2+} binding to the Ca^{2+}-specific sites I and II of troponin C [12], explaining why calmodulin can replace troponin C in the activation of actomyosin ATPase [13]. In contrast, the high affinity Ca^{2+}–Mg^{2+} sites of troponin C and parvalbumin domains III and IV [12,14,15], described as 'relaxing sites', appear to be substantially different in ion-binding and function from the homologous calmodulin domains III and IV.

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References

[1] Cheung, W. Y. (1980) Science 207, 19–27.
[2] Means, A. R. and Dedman, J. R. (1980) Nature 285, 73–77.
[3] Klee, C. B. (1977) Biochemistry 16, 1017–1024.
[4] Seamon, K. B. (1980) Biochemistry 19, 207–215.
[5] Kilhoffer, M. C., Demaille, J. G. and Gerard, D. (1980) FEBS Lett. 116, 264–272.
[6] Watterson, D. M., Sharief, F. and Vanaman, T. C. (1980) J. Biol. Chem. 255, 962–975.
[7] Demaille, J. G., Haiech, J. and Goodman, M. (1980) in: Protides of the Biological Fluids, vol. 28, in press.
[8] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. Tokyo 87, 1313–1320.
[9] Vanaman, T. C. and Sharief, F. (1979) Fed. Proc. FASEB 38, 788.
[10] Autric, F., Ferraz, C., Kilhoffer, M. C., Cavadore, J. C. and Demaille, J. G. (1980) Biochim. Biophys. Acta in press.
[11] Cartaud, A., Ozon, R., Walsh, M. P., Haiech, J. and Demaille, J. G. (1980) J. Biol. Chem. 255, in press.
[12] Potter, J. D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628–4633.
[13] Amphlett, G. W., Vanaman, T. C. and Perry, S. V. (1976) FEBS Lett. 72, 163–168.
[14] Haiech, J., Derancourt, J., Pechère, J. F. and Demaille, J. G. (1979) Biochemistry 18, 2752–2758.
[15] Goodman, M., Pechère, J. F., Haiech, J. and Demaille, J. G. (1979) J. Mol. Evol. 13, 331–352.