Journal of Fluorescence, Vol. 3, No. 1, 1993

Asymmetry of Calmodulin Revealed by Peptide Binding

Estelle Leclerc,[1] Liliane Leclerc,[1] and Michael C. Marden[1]

Received November 5, 1992; revised February 5, 1993; accepted February 5, 1993

The binding of amphiphilic peptides to calmodulin has been studied using fluorescence energy transfer techniques. Calmodulin has no tryptophan residues but possesses two tyrosines (at positions 99 and 138) in the C-terminal half of the protein. The peptides have a single tryptophan which serves as energy acceptor for the protein tyrosine fluorescence. For the binding of mastoparan or peptide Baa17, with a tryptophan at position 3, the observed quenching of the tyrosine fluorescence of over a factor of 2 corresponds to an average tyrosine–tryptophan distance of less than 14 Å. These results indicate that the peptides binds preferentially with the tryptophan in the C-terminal half of the protein.

KEY WORDS: Calmodulin; asymmetry; peptide binding; energy transfer.

INTRODUCTION

Calmodulin (CaM) possesses a rather symmetric structure, containing a globular domain on each side of a central helix [1]. The two domains show a large homology and each contains two binding sites for calcium. Calcium ions induce a change in conformation in calmodulin, which in turn activates various "target" proteins [2]. The binding of calcium apparently "opens" the protein and exposes the central helix and a hydrophobic pocket in each domain [1]. Several other molecules (such as trifluoroperazin) and target peptides have been reported to bind as well [3–5].

NMR studies indicate that the peptide Baa17 binds in one of the hydrophobic domains of the protein [6]. This leaves at least four possible orientations of Baa17 within the peptide–calmodulin complex. Since both tyrosine residues of the protein are in the same (C-terminal) domain, and calmodulin has no tryptophan residues, it appeared to be an ideal case for observing fluorescence energy transfer between tyrosine and tryptophan.

Previous studies have documented the efficiency of transfer from tyrosine to tryptophan residues [7]. Since the overlap between tyrosine fluorescence and tryptophan absorbance is weak, and the tyrosine quantum yield is low, a distance of typically 15 Å is required to decrease the tyrosine fluorescence by a factor of two. Since the size of each domain of calmodulin is 20 Å and the interdomain distance (center to center [1]) is over 30 Å, a preferential location of tryptophan residue in the C-terminal domain should be observable by the quenching of the calmodulin tyrosine fluorescence. In this study we use five peptides which bind with a high affinity to calmodulin: two that have the tryptophan at position 3; two that lack the tryptophan and serve as controls; and melittin, which has the tryptophan at position 19.

EXPERIMENTAL

Calmodulin was prepared from bovine brain acetone powder (Sigma) following methods described previously [8,9]. The protein concentration was determined from the absorption band at 276 nm with $\epsilon = 3740\ M^{-1}\ cm^{-1}$ [10].

The 17-residue peptide Baa17 was a gift from the Pasteur Institute [6]. Both Baa17 and *Polistes* mastoparan (Sigma), a peptide with 14 residues, have a single

[1] INSERM U299, Hôpital de Bicêtre, 94275 Le Kremlin Bicêtre, France.

1053-0509/93/0300-0045$07.00/0

tryptophan at position 3; an absorption coefficient of 5400 M^{-1} cm^{-1} at 280 nm was estimated for these peptides. Sample concentrations were determined from the UV absorption spectra on a SLM-Aminco (DW2000) spectrometer.

Melittin (Boerhinger), a 26-residue peptide with a tryptophan at position 19, ϵ = 5470 M^{-1} cm^{-1} at 280 nm [11], also binds to calmodulin with a high affinity [11–13].

We also studied the effect of binding of two peptides without tryptophan or tyrosine residues: peptide pPKII (Bachem) corresponds to the fragment (290–309) of the calmodulin-dependent protein kinase II [14]; mastoparan (Sigma) is a wasp venom [15]. For these two peptides the concentrations were based on the weight prepared.

The fluorescence spectra were measured on an SLM-Aminco 8000 spectrofluorometer. The present study required a determination of the tyrosine yield in the presence of the peptide containing a tryptophan residue. The method used is to measure the fluorescence emission spectrum using excitation wavelengths of both 275 nm (which excites both tyrosine and tryptophan residues) and 295 nm, which selectively excites the tryptophan residue. The tyrosine contribution can be recovered by the difference between the normalized spectra.

The solvent spectrum under the same conditions was subtracted from each sample spectrum. Note that this is important, since the Stokes' line varies with the excitation wavelength and occurs near the tyrosine emission peak for excitation at 275 nm. Although the intensity for the Stokes's line was less than 5% of the calmodulin signal, it becomes important for highly quenched systems, and failure to account for this solvent contribution will cause an overestimation of the final tyrosine fluorescence. The corrected spectra were then normalized (by a factor c) to match the fluorescence intensity above 380 nm, where the tryptophan fluorescence dominates. Thus, to obtain the tyrosine emission spectrum (above 300 nm), the overall calculation at each wavelength is

$$F(\text{tyr}) = [F_{275}(\text{CaM + Baa17}) - F_{275}(\text{solvent})] - c[F_{295}(\text{CaM + Baa17}) - F_{295}(\text{solvent})] \quad (1)$$

where the subscripts refer to the excitation wavelength (nm).

The experimental conditions were the same for all spectra, except for the change in excitation wavelength. The monochromator slits were set at 1-nm resolution for both excitation and emission in order to obtain the tyrosine peak near 305 nm despite excitation at 295 nm. Quartz cuvettes, 4 × 10 mm, were used with excitation along the 4-mm axis; absorption spectra were measured along the 10-mm path.

RESULTS

The fluorescence emission spectra for calcium bound calmodulin, with and without peptide Baa17, are shown in Fig. 1. As expected, the tryptophan fluorescence dominates when both tyrosine and tryptophan residues are present, due to both the higher absorbance coefficient and a higher fluorescence yield. Despite the larger contribution of the tryptophan residue, a decrease in the overall emission intensity can be observed near 290 nm where there is little tryptophan contribution. This is direct evidence for the quenching of the tyrosine fluorescence. As a first approximation, one can simply take the ratio of the emission spectra of calmodulin to that of CaM + peptide (after subtraction of the solvent spectrum from each curve). This method provides a minimum quenching factor, since the tryptophan contribution is not subtracted off; a quenching factor of 2.2 (± 0.2) was observed for the average of seven independent measurements with Baa17. Note that a shift in the calmodulin emission spectrum due to the peptide binding would influence the observed ratio calculated by this method.

In order to obtain the fluorescence spectrum of the calmodulin tyrosines in the presence of the peptide, the peptide tryptophan fluorescence must be subtracted. The tryptophan emission can be isolated by excitation at 295 nm, where excitation of the tyrosine residues is negligible. After the solvent spectra were subtrated for each

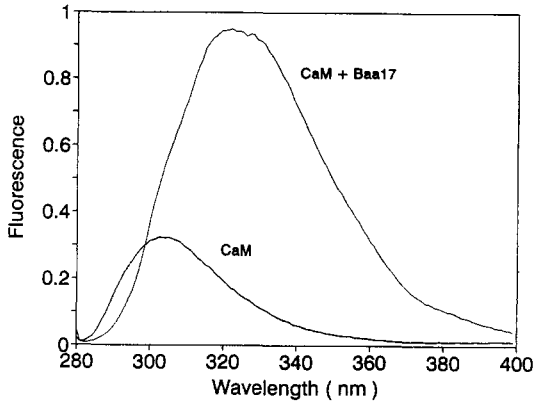

Fig. 1. Fluorescence emission spectra of calcium-bound calmodulin (CaM), with and without the peptide Baa17. Excitation was at 275 nm for samples in 50 mM Tris buffer at pH 7.4, 25°C. Concentrations were 7 μM calmodulin, 9 μM peptide, and 1 mM calcium.

spectrum, and after normalization of the spectra (275- and 295-nm excitation) in the region above 380 nm, subtraction of the spectra yields the calmodulin fluorescence in the presence of the peptide (Fig. 2). The average decrease in the calmodulin tyrosine fluorescence, due to the binding peptide Baa17, is a factor of 2. The quenching factor was determined from the peak intensity values near 305 nm, rather than integration of the spectra, since the full tyrosine spectrum could not be obtained.

Similar results were obtained with *Polistes* mastoparan (tryptophan at position 3); a quenching factor of 2.3 was observed. Melittin was also used for comparison; in this case the peptide of 26 residues has a single tryptophan at position 19. As shown in Fig. 2, an intermediate quenching level was observed, indicating a larger tyrosine–tryptophan distance, as estimated on the right-hand axis. The quenching factors for the peptides are summarized in Table I.

The energy transfer calculations, based on an R_0 of 14.2 Å for tyrosine-99 (quantum yield Φ = 0.095) and 13.2 Å for tyrosine-138 (Φ=0.061 [7,16]), imply an average donor–acceptor distance of approximately R_0 = 14 Å for a quenching of a factor of 2. These values indicate that the peptide tryptophan is in fact in the same half of the protein as the two tyrosine residues. The right-hand axis in Fig. 2 gives the distance (R) necessary for the observed quenching for the Förster energy transfer [17]: $Q_a/Q = 1 + (R_0/R)^6$, where Q_a is the fluorescence intensity in the absence of quenching and R_0 = 14 Å.

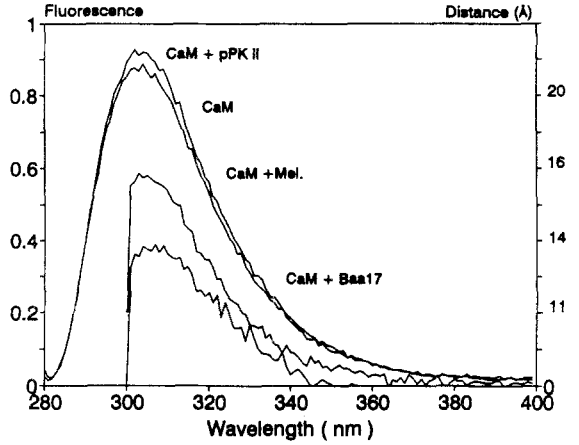

Fig. 2. Tyrosine emission spectra for calcium-bound calmodulin (CaM), with and without the peptide indicated. Experimental conditions: 5 μM calmodulin, 6 μM for the peptides, 1 mM calcium, 25°C, 50 mM Tris buffer at pH 7.4. In the presence of peptide, the tyrosine spectrum was calculated as the difference in emission at two excitation wavelengths (275 minus 295 nm), after subtraction of solvent and normalization of the spectra above 380 nm where the tryptophan fluorescence dominates. Control measurements were made using a similar peptide without a tryptophan residue (curve pPKII) and melittin (Mel), which has one tryptophan at position 19. The right-hand axis indicates the tyrosine–tryptophan distance necessary to obtain the observed fluorescence intensity, based on the quenching factor $Q_a/Q = 1 + (R_0/R)^6$ with R_0 = 14 Å.

The errors in the spectroscopic measurements and subsequent corrections are only a few percent. The largest error (±10% in the quenching factor) was in the sample-to-sample reproducibility; this error is still small compared to the quenching factor and does not affect the conclusions. However, other factors need to be considered. For example, it is known that the calmodulin tyrosine fluorescence intensity decreases by over a factor of two when the calcium is removed [16]. Thus environmental factors could account for part of the decrease and further controls were performed.

Control 1. Free DL-tryptophan was added to calmodulin samples. This tests essentially for the inner filter effects, since the absorption properties of Baa17 and free tryptophan are similar. There was no change in the free tryptophan fluorescence emission, indicating that it did not bind to the protein. Under the same conditions used for the interaction calmodulin–peptide, the free tryptophan did not decrease the calmodulin fluorescence (data not shown), indicating negligible inner filter effects.

Control 2. The 20-residue peptide pPKII derived from calmodulin-dependent protein kinase II, without tryptophan residues, was also tested. Since this peptide is similar to Baa17, it should provide a similar environment, but without the energy transfer. The fluorescence spectra of calmodulin showed no change in peak emission wavelength and a small increase in fluorescence intensity upon binding of pPKII (Fig. 2). Measurements were also made with mastoparan (without tryptophan); in this case there was no increase in the calmodulin tyrosine fluorescence.

DISCUSSION

The NMR study [6] indicated that the peptide Baa17 binds in one of the protein hydrophobic pockets. The present results localize the peptide with the tryptophan residue in the C-terminal domain of calmodulin. Due to the apparent symmetry of the protein and peptide, one would expect a more random binding. The large decrease, a factor of 2 (R < 14 Å), in the calmodulin (tyrosine) fluorescence due to quenching by the peptide

Table I. Sequences of the Calmodulin-Binding Peptide Studies[a]

Peptide	Sequence	Q_a/Q
pPKII	L-K-K-F-N-A-R-R-K-L-K-G-A-I-L-T-T-L-A	0.95
Mastoparan	I-N-L-K-A-L-A-A-L-A-K-K-I-L	1.0
Melittin	G-I-G-A-V-L-K-V-L-T-T-G-L-P-A-L-I-S-W-I-K-R-K-R-E-E	1.4
Baa17	L-K-W-K-K-L-L-K-L-L-K-K-L-L-K-L-G	2.1
Polistes Mastoparan	V-D-W-K-K-I-G-Q-H-I-L-S-V-L	2.3

[a]The quenching factor is the ratio of the peak tyrosine fluorescence of calmodulin alone (Q_a) to that of calmodulin with the peptide (Q); the largest error (10%) was in the sample-to-sample variation.

tryptophan indicates a breaking of this symmetry. These results are consistent with photolabeling studies [18] which reported a cross-linking of a marker at position 3 of Baa17 (substitution for the tryptophan) with the methionine-144 residue in the C-terminal of calmodulin.

From the crystallographic coordinates for calcium-bound calmodulin [1], one can show the positions of the tyrosines relatives to the central helix and the N- and C-terminal domains (Fig. 3, using the program Molscript [19]). A donor–acceptor distance of less than 15 Å would be expected for the peptide with the tryptophan-containing end (N terminal) in the C-terminal protein pocket. A distance of over 25 Å would be expected for peptide binding in the calmodulin N-terminal pocket. The predicted quenching factors would be a factor of 2 for the first case, 1.2 for the second, and 1.4 for an equal mixture of the two orientations. The fluorescence energy transfer data are thus consistent with the peptide binding preferentially with its tryptophan in the protein C-terminal domain.

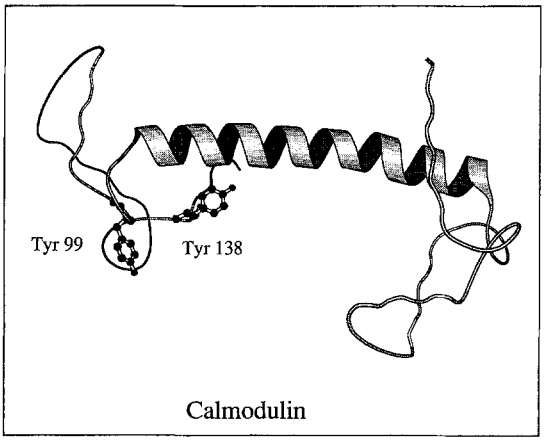

Fig. 3. Structure of calcium-bound calmodulin [1], showing the two tyrosine residues; the end-to-end distance is 65 Å. The central helix (residues 65–92) is presented in helical form; the N- and C-terminal domains are shown in the coil representation (using Molscript [17]).

As expected, the binding of melittin (tryptophan at position 19) shows a smaller quenching factor. There is probably a significant quenching of tyrosine-138, since it is more centrally located (Fig. 3). If the peptide Baa17 (of total length 25 Å) were bound with its C terminal in the protein C-terminal pocket, one would expect a lower quenching factor, as for melittin.

Control experiments are important for this type of study, since the fluorescence intensity depends on the local environment of the residue, in addition to the energy transfer rates. Fortunately additional peptides are available to provide a similar binding. The results with pPKII and mastoparan (without tryptophan) show a null result, while melittin (with the tryptophan farther in the sequence) shows a reduced quenching (Fig. 2). Baa17 and *Polistes* mastoparan, both with the tryptophan at position 3, show similar quenching factors of over a factor of 2. These results are consistent with quenching by energy transfer, rather than a change in environment.

In a recent crystallographic study, the binding of a related peptide from chicken smooth muscle myosin light-chain kinase also showed a specific binding orientation [20]. In this case the tryptophan residue at position 5 was located in the C-terminal hydrophobic pocket of calmodulin. Solution studies using NMR techniques confirmed this result [21].

The same peptide–protein orientation has been reported for a 26-residue fragment of the skeletal muscle myosin light-chain kinase [22]. There is thus a growing evidence for a number of peptides which bind with their N terminal preferentially in the calmodulin C terminal. The present results, under solution conditions at a low protein concentration, indicate the same orientation for the binding of Baa17 or mastoparan to calmodulin.

ACKNOWLEDGMENTS

We thank Dr. O. Bârzu of the Pasteur Institute for supplying the peptide Baa17; we are grateful to Drs. C. T. Craescu and C. Poyart for useful discussions; we thank Dr. J. Kister for aid in the use of the Molscript program [19].

REFERENCES

1. Y. S. Babu, C. E. Bugg, and W. J. Cook (1988) *J. Mol. Biol.* **204,** 191–204.
2. P. Cohen and C. B. Klee, (Eds.) (1988) *Calmodulin. Molecular Aspects of Cellular Recognition, Vol. 5,* Elsevier Biomedical Press, Amsterdam.
3. W. F. DeGrado, F. G. Prendergast, H. R. Wolfe Jr., and J. A. Cox (1985) *J. Cell. Biochem.* **29,** 83–93.
4. J. A. Cox, M. Comte, J. E. Fitton, and W. F. DeGrado (1985) *J. Biol. Chem.* **260,** 2527–2534.
5. K. T. O'Neil, H. R. Wolfe Jr., S. Erickson-Viitanen, and W. F. DeGrado (1987) *Science* **236,** 1454–1456.
6. B. Prêcheur, H. Munier, J. Mispelter, O. Bârzu, and C. T. Craescu (1992) *Biochemistry* **31,** 229–236.
7. M. C. Kilhoffer, D. M. Roberts, A. Adibi, D. M. Watterson, and J. Haiech (1989) *Biochemistry* **28,** 6086–6092.
8. D. L. Newton, M. H. Krinks, J. B. Kaufman, J. Shiloach, and C. B. Klee (1988) *Prep. Biochem.* **18,** 247–259.
9. R. Gopalakrishna and W. B. Anderson (1982) *Biochem. Biophys. Res. Comm.* **104,** 830–836.
10. D. J. Wolff, P. G. Poirier, C. O. Brostrom, and M. A. Brostrom (1977) *J. Biol. Chem* **252,** 4108–4117.
11. Y. Maulet and J. A. Cox (1983) *Biochemistry* **22,** 5680–5686.
12. M. Comte, Y. Maulet, and J. A. Cox (1983) *Biochem. J.* **209,** 269–272.
13. C. E. Dempsey (1990) *Biochim. Biophys. Acta* **1031,** 143–161.
14. M. E. Payne, Y. L. Fong, T. Ono, R. J. Colbran, B. E. Kemp, T. R. Soderling, and A. R. Means (1988) *J. Biol. Chem* **263,** 7190–7195.
15. D. A. Malencik and S. R. Anderson (1983) *Biochem. Biophys. Res. Comm.* **114,** 50–56.
16. M. C. Kilhoffer, J. G. Demaille, and D. Gerard (1981) *Biochemistry* **20,** 4407–4414.
17. T. Förster (1959) *Disc. Faraday Soc.* **27,** 7–17.
18. K. T. O'Neil, S. Erickson-Viitanen, and W. F. DeGrado (1989) *J. Biol. Chem.* **264,** 14571–14578.
19. P. J. Kraulis (1991) *J. Appl. Crystollogr.* **24,** 946–950.
20. W. E. Meador, A. R. Means, and F. A. Quiocho (1992) *Science* **257,** 1251–1255.
21. S. M. Roth, D. M. Schneider, L. A. Strobel, M. F. A. Van Berkum, A. R. Means, and A. J. Wand (1992) *Biochemistry* **31,** 1443–1451.
22. M. Ikura, G. M. Clore, A. M. Gronenborn, G. Zhu, C. B. Klee, and A. Bax (1992) *Science* **256,** 632–638.