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The Interaction of Calmodulin With Fluorescent and Photoreactive Model Peptides: Evidence for a Short Interdomain Separation

K.T. O'Neil and W.F. DeGrado
E.I. du Pont de Nemours and Company, Central Research and Development Department, Experimental Station, P.O. Box 80328, Wilmington, Delaware 19880-0328

ABSTRACT **Calmodulin is known to bind target enzymes and basic, amphiphilic peptides in a Ca^{2+}-dependent manner. Recently, we introduced a photoaffinity label, *p*-benzoylphenylalanine (Bpa), into the sequence of a model, α-helical, calmodulin-binding peptide. When the Bpa residue was introduced at the third position of the peptide, Met-144 on the C-terminal domain of calmodulin was labeled, whereas when the photolabel was placed at the thirteenth position, Met-71 on the N-terminal domain was labeled. Assuming that both peptides bind in similar orientations, these results are not consistent with the crystal structure of calmodulin, in which the domains are held at a significant distance from one another by a long α-helical segment. To test the assumption that bo th peptides bind in similar orientations, we have synthesized a calmodulin-binding peptide with the photolabel in both the third and the thirteenth positions. Upon photolysis, this peptide forms a cross-link between Met-71 and Met-124 on the N- and C-terminal domains, respectively. Furthermore, a peptide with a Bpa in the thirteenth position and a Trp residue in the third position was also synthesized. After photocross-linking the Bpa residue of this peptide to Met-71 of calmodulin, it could be shown that the fluorescence properties of the Trp residue were consistent with its side chain being buried in a hydrophobic pocket on the C-terminal domain of calmodulin. These data indicate that, when complexed with basic, amphiphilic peptides, calmodulin can adopt a conformation in which its two domains are significantly closer than in the crystal structure of the uncomplexed protein.**

Key words: calmodulin, peptides, fluorescence spectroscopy, photoaffinity labeling

INTRODUCTION

Calmodulin is an intracellular calcium-binding protein responsible for the regulation of a variety of structurally and functionally diverse enzymes.[1] The molecular basis for calmodulin's ability to activate such a host of enzymes is an intriguing problem in molecular recognition. On one hand, the promiscuity of the interaction would argue for a nonspecific association, and yet calmodulin shows considerable sequence conservation among proteins from different sources. Furthermore, calmodulin binds target enzymes quite tightly (with nanomolar dissociation constants) consistent with a more specific interaction.

Recent chemical, biophysical, and structural studies have shed light on the molecular mechanism of target enzyme activation.[2,3] Studies with synthetic model peptides have shown that calmodulin has a high affinity for basic, amphiphilic α-helices and this structural feature has been found in many if not all calmodulin-binding proteins (reviewed in references 2, 3). The crystal structure of calmodulin[4,5] suggests a molecular rationale for calmodulin's ability to interact with this structural feature. It shows two homologous domains each containing two EF hand calcium-binding motifs. Both domains have a large, hydrophobic patch flanked by acidic residues that singly or in combination might be capable of binding a basic amphiphilic helix. In the crystal structure the domains are held at some distance from one another by a long α-helical segment, but solution data suggest that this helix might be disordered or bent allowing a much closer proximity of the two domains. Low angle X-ray scattering studies of calmodulin in solution are consistent with a structure in which the individual domains have approximately the same radii as in the crystal structure but in which the domains are 5–10 Å closer together than in the crystal structure.[6] Furthermore, NMR studies indicate that when calmodulin binds amphiphilic peptides, changes occur in the chemical shifts and calcium-binding properties of both domains.[7,8] Also, mutants of calmodulin in which the two domains have been covalently tethered close to one another through the introduction of specific

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Address reprint requests to William F. DeGrado, E.I. du Pont de Nemours and Company, Central Research and Development Department, Experimental Station, P.O. Box 80328, Wilmington, DE 19880-0328.

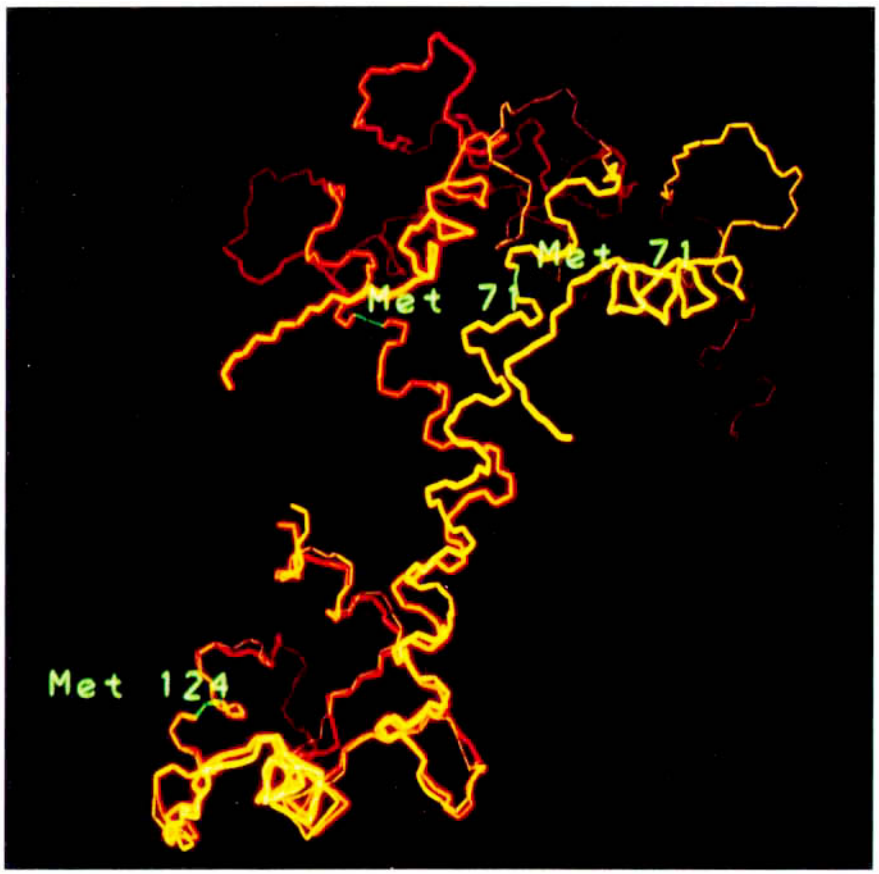

Fig. 1. Comparison of the crystal structure of calmodulin with a model containing a bend in the central helix. Only backbone atoms are shown; the crystal structure is shown in orange and the model in yellow. Coordinates for the crystal structure are from the 2.2 Å map described by Babu and co-workers[4]; coordinates for the "bent" model are from the structure described by Persechini and Kretsinger.[3]

cross-links retain high activity toward target enzyme activation.[9]

Figure 1 illustrates the crystal structure of calmodulin[4,5] and a proposed structure[3] for calmodulin as it is believed to exist when in a complex with the calmodulin-binding domain of myosin light chain kinase (MLCK). By bending the central helix it is possible to bring the two domains into proximity so that both can simultaneously interact with an α-helical peptide.

Recent photoaffinity labeling studies using photoreactive derivatives of calmodulin-binding peptides have provided indirect support for this structural hypothesis. We have developed a photoreactive amino acid *p*-benzoylphenylalanine,[10] Bpa, and this residue has been incorporated into the third and thirteenth positions of a 17-residue amphiphilic calmodulin-binding peptide (Table I). When the photolabel was in the third position of the peptide as in Bpa_3, Met-144 on the C-terminal domain of calmodulin was uniquely labeled, whereas with Bpa_{13}, the labeling occurred primarily at Met-71 on the C-terminal domain.[11] These two Met residues are approximately 30 Å distant in the X-ray structure of calmodulin (C_α to C_α), a distance that cannot reasonably be spanned by the peptide in a helical conformation. This indicates that either the two peptides bind to calmodulin at different sites, or that the two domains are closer together than in the crystal structure of the uncomplexed protein and form a single binding site as in the model in Fig. 1.

In the present work we describe experiments designed to distinguish between these two possibilities. A peptide, $Bpa_{3,13}$, was synthesized in which Bpa was incorporated at both the third and thirteenth positions (Table I). We find that $Bpa_{3,13}$ specifically photocross-links both domains of calmodulin. There are, however, limitations associated with the interpretation of this experiment. It is possible that calmodulin spends only a fraction of time in a conformation that bridges the domains, but that the equilibrium of the bridging interaction is pulled toward this conformation by covalent bond formation between calmodulin and the peptide.

TABLE I. Amino Acid Sequences of Calmodulin-Binding Peptides

Trp_3	Leu Lys Trp Lys Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu Gly
Bpa_3	Leu Lys Bpa Lys Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu Gly
Bpa_{13}	Leu Lys Leu Lys Lys Leu Leu Lys Leu Leu Lys Lys Bpa Leu Lys Leu Gly
$Bpa_{3,13}$	Leu Lys Bpa Lys Lys Leu Leu Lys Leu Leu Lys Lys Bpa Leu Lys Leu Gly
Trp_3–Bpa_{13}	Leu Lys Trp Lys Lys Leu Leu Lys Leu Leu Lys Lys Bpa Leu Lys Leu Gly

To minimize this limitation, we have also synthesized a peptide with the photolabel at the thirteenth position and a fluorescent label in the third position. Because calmodulin does not contain a Trp residue in its sequence it is possible to use Trp as the fluorescent probe. After photocross linking of the peptide to calmodulin (which tethers the peptide to Met-71), the fluorescence properties of the peptide photoadduct when compared to those for the peptide free in solution will give information about the environment of the Trp near its N-terminus. If the Trp is bound to the hydrophobic patch on the C-terminal lobe of calmodulin, the maximum for fluorescence emission will occur at relatively short wavelengths, the anisotropy will be relatively high, and the accessibility to quenching by acrylamide will be relatively low.[12] In contrast, if the fluorescent probe is spending only a fraction of the time at the hydrophobic surface the fluorescence properties will reflect a much smaller overall change when compared to the properties measured in the absence of calmodulin.

A precise prediction of the Trp fluorescence properties to be expected for a given binding orientation was made possible by our earlier work involving a series of peptides with a Trp residue placed at each possible position in a 17-residue amphiphilic peptide.[13] The fluorescence properties of this series of peptides were found to be periodic in a manner consistent with an α-helical conformation. In particular, we discovered that when Trp was in the third position (Trp_3, Table I) the fluorescence properties were by far the most affected by calmodulin binding. Our photoaffinity labeling results together with computer modeling indicated that this particularly strong perturbation of the fluorescence parameters was a result of the Trp residue occupying a deep hydrophobic crevice on the C-terminal lobe of calmodulin.[11] We therefore anticipated that if the peptide were indeed binding to both lobes of calmodulin, the Trp_3–Bpa_{13} photoadduct would have fluorescent properties nearly identical to those found for the Trp_3:calmodulin complex.

MATERIALS AND METHODS

Materials

Bovine brain calmodulin was obtained from Pharmacia/LKB (Piscataway, NJ) and *S. aureus* V8 protease was from ICN Biochemicals (Cleveland, OH); both were used as received with no further purification. Boc-Bpa was the generous gift of J. C. Kauer. [^{3}H]Acetic anhydride was purchased from Du Pont NEN (Billerica, MA). Boc-protected amino acids and amino acid resins were purchased from NovaBiochem (Laufelfingen, Switzerland), Bachem (Torrance, CA), or Advanced ChemTech (Louisville, KY).

Synthesis and Purification of $Bpa_{3,13}$ and Trp_3–Bpa_{13} Peptides

Both peptides were synthesized manually on Boc-Gly-Merrifield resin analogously to other Bpa-containing peptides.[11] The Trp side chain was unprotected in the case of Trp_3–Bpa_{13}. $Bpa_{3,13}$ was acetylated with [^{3}H]acetic anhydride and Trp_3–Bpa_{13} with unlabeled acetic anhydride as described previously.[11] Crude peptides were purified in a single step by reverse-phase HPLC using a Vydac C_{18} preparative column (purchased from the Nest Group, Southboro, MA). A gradient from 39% to 45% aqueous acetonitrile (containing 0.1% TFA) in 21 minutes yielded chromatographically pure $Bpa_{3,13}$; a gradient of 35% to 46% aqueous acetonitrile in 21 minutes yielded pure Trp_3–Bpa_{13}. Overall yields for the peptides were 16% for Trp_3–Bpa_{13} and 6.5% for $Bpa_{3,13}$. Amino acid analysis for $Bpa_{3,13}$: Leu 7.01 (7), Lys 7.07 (7), and Gly 0.93 (1) and Trp_3–Bpa_{13}: Leu 7.03 (7), Lys 6.96 (7), and Gly 1.01 (1) as well as fast atom bombardment mass spectrometry $Bpa_{3,13}$ $(M + H)^+ = 2308.5$ (calc. 2309.2) and Trp_3–Bpa_{13} $(M + H)^+ = 2245.0$ (calc. 2244.4) indicated that the desired peptides had been obtained.

Synthesis and Purification of MLCK-Cys Peptide

The calmodulin-binding domain from smooth muscle myosin light chain kinase with a C-terminal cysteine added to allow attachment to a solid support was synthesized analogously to other MLCK peptides.[11] The peptide, RRKWQKTGHAVRLIGRLSSC, was purified to homogeneity by reverse-phase HPLC using a gradient of 15–24% acetonitrile (containing 0.1% TFA) in 30 minutes. Amino acid analysis Thr 1.1 (1), Ser 1.3 (2), Glx 1.0 (1), Gly 1.9 (2), Ala 2.0 (2), Val 1.1 (1), Ile 1.0 (1), Leu 1.1 (1), His 0.9 (1), Lys 1.8 (2), Arg 4.1 (4), and FAB mass spectrometry $(M + H)^+ = 2310.2$ (calc. 2310.3) indicated that the desired peptide had been obtained.

Photolabeling of Calmodulin

Photolabeling was performed as described previously[11] with the following modifications. Photoly-

sis of $Bpa_{3,13}$ with calmodulin yielded the most homogeneous product when the reaction mixture was kept frozen for the course of the photolysis. Solutions for photolysis were divided into small volumes in individual wells of 24-well tissue culture plates and frozen in liquid nitrogen. Samples were kept on dry ice during the photolysis for the entire course of the reaction. For reverse-phase HPLC analysis to determine the extent of reaction, samples were thawed quickly and refrozen in liquid nitrogen after removal of an aliquot for analysis. Using an analytical Vydac C_{18} column, separation of photoadduct and starting species was achieved with a gradient from 27 to 47% acetonitrile (containing 0.1% TFA) in 45 minutes.

Enzymatic Digestions

S. aureus V8 digestion of photoadducts was performed as described previously.[11]

Purification of Trp_3–Bpa_{13} Photoadduct

For fluorescence experiments, the photoadduct was purified by FPLC using a Mono Q (Pharmacia/LKB, Piscataway, NJ) column and a linear gradient of ammonium acetate from 0 to 0.5 M in 50 minutes (both A and B buffers contained 0.5 mM $CaCl_2$). Fractions were collected and analyzed by HPLC as described above to identify the fractions containing homogeneous photoadduct. The pooled fractions were stored frozen, and the concentration of the photoadduct was determined by amino acid analysis.

Fluorescence Spectroscopy

Fluorescence measurements were performed on a Spex Fluorolog 222 fluorometer. Anisotropy, acrylamide quenching, and fluorescence emission spectra were measured for 10 μM peptide or peptide:calmodulin photoadduct as described previously.[11] All measurements were made in 10 mM Tris, 0.5 mM $CaCl_2$, pH 7.5.

Affinity Measurements

Determinations of the affinity of Trp_3–Bpa_{13} for calmodulin were made analogously to the method we have described previously using peptide immobilized on resin support[14,15] with the following exceptions. The calmodulin-binding domain from smooth muscle myosin light chain kinase with a cysteine residue added to the C-terminus was coupled to Thiopropyl Sepharose 6B (obtained from Pharmacia/LKB, Piscataway, NJ). Free thiol peptide (0.1 μmol) (determined quantitatively using Ellman's reagent[16]) was added to 0.5 g Thiopropyl Sepharose 6B in 5 ml 0.1 M Tris, pH 8.0. After 20 minutes rotating end over end at room temperature, the resin was filtered and washed with several aliquots of pH 8.0 buffer to a total filtrate volume of 12 ml. Using an extinction coefficient of 8080 M^{-1} at 343 nm for the 2-thiopyridine displaced from the resin, it was determined that 0.075 μmol of peptide had been coupled to the Sepharose. Using an estimate of 1 g/3 ml for the resin, a substitution level of approximately 0.05 μmol/ml resin was calculated. The resin was stored lyophilized in the freezer at −20°C. Of 25 μM peptide 50 μl was solubilized in 30 mM imidazole, 135 mM KCl, 0.1 mg/ml BSA, 1 mM $CaCl_2$, pH 7.0 and was serially diluted into 11 Eppendorf microcentrifuge tubes; a twelfth tube contained only buffer as a blank. A 2.85 mg/ml suspension of peptide-conjugated resin containing 75 nM calmodulin trace labeled with [^{3}H]acetic anhydride was preincubated for 5 minutes at room temperature. Of this suspension 200 μl was added to each tube and rotated end over end for 5 minutes at room temperature before centrifuging for 1 minute in a microcentrifuge. The top 100 μl of each sample was counted and tritium cpm plotted as a function of the logarithm of peptide concentration. The dissociation constant was calculated by comparison of the curve for Trp_3–Bpa_{13} with that for the standard, Trp_3. The affinity measurement for $Bpa_{3,13}$ was not possible as the peptide was labeled with tritium, which would interfere with measurement of the tritium-labeled calmodulin used in the assay.

Miscellaneous

Fast atom bombardment mass measurements were made on a VG ZAB-E mass spectrometer fitted with an Ion Tech gun using xenon as the ionizing gas. Amino acid analyses were obtained using a Beckman Amino Acid Analyzer 119 Cl ion-exchange system with ninhydrin detection. N-terminal sequencing analyses were obtained on an Applied Biosystems 470A Protein/Peptide Sequencer interfaced with an Applied Biosystems 120A PTH analyzer.

RESULTS

Photolabeling of Calmodulin

The time course for the reaction of Trp_3–Bpa_{13} with calmodulin is analogous to that described previously for Bpa_3 and Bpa_{13}.[11] The reaction was essentially complete after 2 hours as judged by the conversion of greater than 90% of the starting calmodulin and peptide to a new peak. Similar results were found for $Bpa_{3,13}$ with the exception that the reaction appears to be sequential; an adduct with only one of the Bpa residues cross-linked can be isolated prior to the formation of the doubly linked adduct. If the reaction is allowed to proceed for 2 hours, the major product formed is a mono-adduct in which the peptide is linked to Met-71 of calmodulin (data not shown). Formation of the doubly linked adduct required a total photolysis time of 4 hours.

Determination of the Binding Affinity of Trp_3–Bpa_{13} for Calmodulin

Trp_3–Bpa_{13} behaves in a manner indistinguishable from Trp_3 in our calmodulin binding assay. As

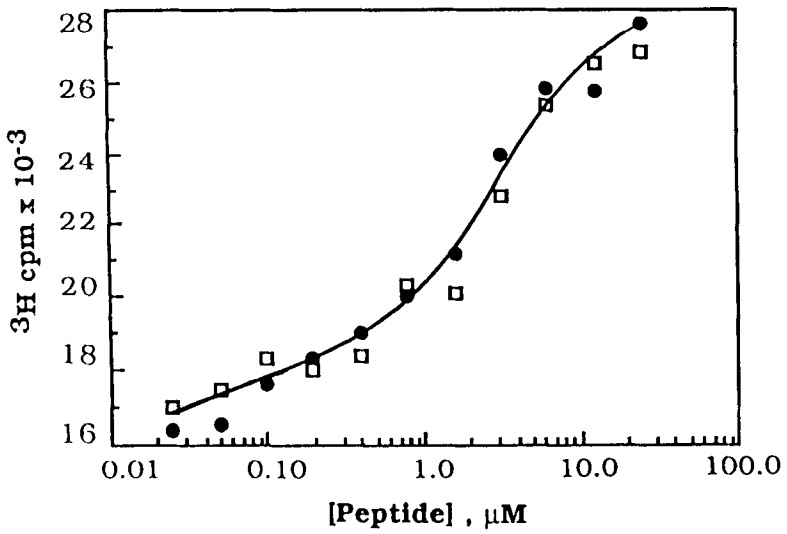

Fig. 2. Binding of ^{3}H-labeled calmodulin by Trp_3 (filled circles) or Trp_3–Bpa_{13} (open squares). MLCK-coupled Thiopropyl Sepharose 6B (200 μl of 1.85 mg/ml) containing 78 nM calmodulin trace labeled with ^{3}H was added to 50 μl of peptide solution. After incubation and centrifugation the calmodulin displaced from the resin was quantitated by scintillation counting the top 100 μl of solution for tritium. Tritium counts in the supernatant are plotted as a function of peptide concentration. Within experimental error, the curves for displacement of calmodulin from the resin are identical for Trp_3 and Trp_3–Bpa_{13}.

illustrated in Figure 2 the partitioning of ^{3}H-labeled calmodulin between MLCK peptide immobilized on Sepharose and Trp_3–Bpa_{13} in solution is exactly the same as that observed for Trp_3. Therefore, K_{diss} for Bpa–Trp is 0.2 nM, the same within experimental error as that calculated previously for Trp_3. This is to be expected based on our earlier results[13] that a Trp residue at position 3 increases the affinity of the peptide for calmodulin but a Trp at position 13 has little effect.

S. aureus V8 Digestion of $Bpa_{3,13}$ Photoadduct

The enzymatic digestion of the $Bpa_{3,13}$ adduct proceeded very slowly in comparison with monoadducts,[11] consistent with a very stable, folded structure for the adduct. Figure 3 illustrates the reverse-phase HPLC UV absorbance and radioactivity profiles for the *S. aureus* V8 digest of the $Bpa_{3,13}$ photoadduct. Three peaks containing significant radioactivity eluted late in the gradient. Each peak was purified to homogeneity by reverse-phase HPLC and identified by amino acid analysis and sequencing. Results for Edman sequence analysis of the three peaks (labeled A, B, and C) are illustrated in Table II. Both peaks A and B showed two different PTH (phenylthiohydantoin) amino acids at each cycle in an approximately one to one molar ratio consistent with their being a doubly cross-linked photoadduct. The sequence information for the peptide eluting at peak A is consistent with that for calmodulin residues 55–84 and 105–140. In addition, there was a gap in the one of the sequences at cycle 17 corresponding to Met-71 in the calmodulin sequence, as well as a second gap at cycle 20, corresponding to Met-124 in the other sequence. No unusual PTH amino acids were observed at these cycles, consistent with the expectation that the labeled Met residue should remain covalently attached to the $Bpa_{3,13}$ peptide absorbed to the filter. A normal value for Met-72 or Ile-125 and all subsequent cycles was observed.

Edman sequencing of the material eluting at peak B indicated that it contained residues 55–84 of calmodulin plus a second sequence beginning with residue 88 of calmodulin. In the first sequence, Met-71 was absent from cycle 17 indicating that this was one of the residues derivatized in the photocross-linking reaction. Although the peptide washed out before the end of the second sequence was reached, amino acid analysis (Table III) indicated that the second sequence spanned residues 88–140 of calmodulin. From the level of Met in the analysis, it can be inferred that the other residue derivatized in the cross-linking reaction was also Met. There are two Met residues in the 88–140 fragment of calmodulin: one at position 109 and one at 124. As Met-109 was observed at normal levels in the Edman sequencing data (Table II), it follows that the derivatized Met was residue 124. Thus, the material eluting at peak B corresponds to the $Bpa_{3,13}$ peptide cross-linked to residues 88–140 plus 55–84 of calmodulin (i.e., peak B is proteolytic precursor to peak A).

A single sequence was observed for the material eluting in peak C indicating that it is a monoadduct

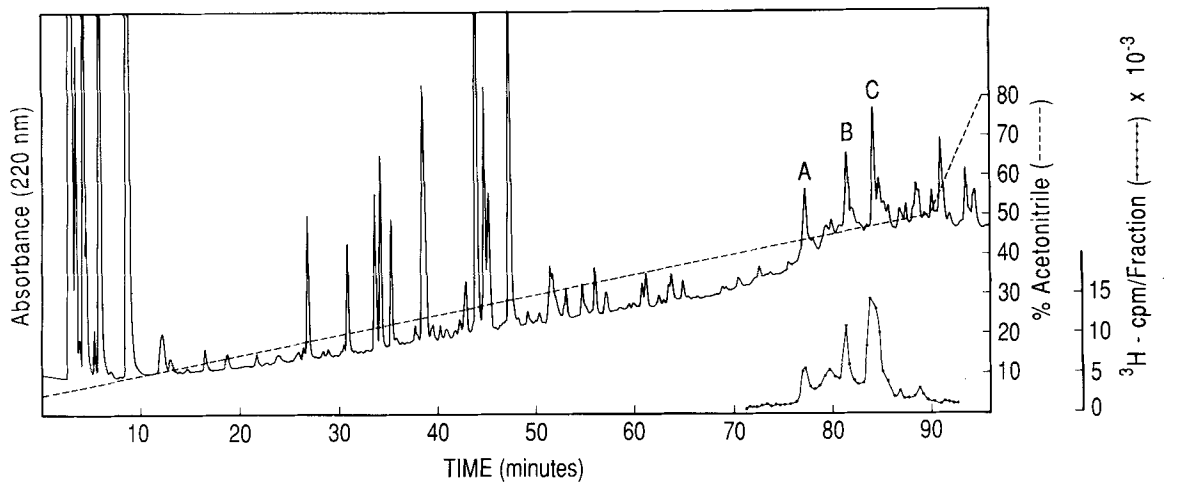

Fig. 3. Reverse-phase HPLC profile of the *S. aureus* V8 digestion of the $Bpa_{3,13}$ photoadduct. The absorbance profile at 220 nm is drawn in a solid line and the acetonitrile gradient in a dashed line. A 40 μl injection was made of a solution containing 1 mg/ml photoadduct digest; the absorbance range was 0.08 with a 1 cm pathlength cell. Fractions were collected at 0.5 minute intervals and counted for ^3H to identify factors containing the labeled peptide. Tritium counts are shown with connected dots below the absorbance profile.

TABLE II. Amino Acid Sequences of V8 Fragments of the $Bpa_{3,13}$ Photoadduct

	PTH amino acid (pmol)		
Cycle	Peak A	Peak B	Peak C
1	Val/Leu (590/478)	Val/Ala (722/667)	Val (771)
2	Asp/Arg (470/180)	Asp/Phe (533/532)	Asp (804)
3	Ala/His (537/91)	Ala/Arg (691/281)	Ala (943)
4	Asp/Val (388/510)	Asp/Val (453/751)	Asp (815)
5	Gly/Met (295/522)	Gly/Phe (420/544)	Gly (583)
6	Asn/Thr (286/366)	Asn/Asp (406/491)	Asn (635)
7	Gly/Asn (195/254)	Gly/Lys (298/410)	Gly (485)
8	Thr/Leu (244/313)	Thr/Asp (471/324)	Thr (588)
9	Ile/Gly (317/165)	Ile/Gly (435/409)	Ile (640)
10	Asp/Glu (288/246)	Asp/Asn (358/292)	Asp (607)
11	Phe/TMK* (256/*)	Phe/Gly (395/171)	Phe (544)
12	Pro/Leu (119/217)	Pro/Tyr (144/249)	Pro (274)
13	Glu/Thr (98/215)	Glu/Ile (173/272)	Glu (249)
14	Phe/Asp (110/194)	Phe/Ser (150/166)	Phe (259)
15	Leu/Glu (71/112)	Leu/Ala (112/244)	Leu (231)
16	Thr/Glu (63/143)	Thr/Ala (100/291)	Thr (191)
17	X/Val (/148)	X/Glu (/187)	X
18	Met/Asp (84/158)	Met/Leu (126/148)	Met (272)
19	Ala/Glu (58/93)	Ala/Arg (78/92)	Ala (200)
20	Arg/X (33/)	Arg/His (66/38)	Arg (93)
21	Lys/Ile (45/115)	Lys/Val (71/134)	Lys (153)
22	Met/Arg (49/44)	Met/Met (227)	Met (198)
23	Lys/Glu (61/74)	Lys/Thr (37/128)	Lys (86)
24	Asp/Ala (37/94)	Asp/Asn (59/72)	Asp (128)
25	Thr/Asp (27/113)	Thr/Leu (19/114)	Thr (103)
26	Asp/Ile (123/75)	Asp/Tly (108/74)	Asp (156)
27	Ser/Asp (29/166)	Ser/Glu (22/64)	Ser (41)
28	Glu/Gly (11/37)	Glu/TMK* (44/*)	Glu (50)
29	Glu (21)	Glu/Leu (49/79)	
30	Glu (32)	Glu/Thr (59/64)	

*TMK is trimethyl-lysine, which was not resolved under in the chromatographic method used to separate PTH amino acids.

TABLE III. Amino Acid Analysis of Peak B of the $Bpa_{3,13}$ Photoadduct V8 Digest

Amino acid	Expected*	Observed
Asx	16	15.3
Thr	5	5.9
Ser	2	1.7
Glx	14	14.1
Gly	8	8.2
Ala	6	6.1
Val	5	4.5
Met†	5	3.2†
Ile	5	4.6
Leu	11	14.2
Tyr	2	1.7
Phe	4	3.5
His	1	0.9
Lys	10	12.2
Arg	5	5.3
Pro	1	1.0

*Calculated for $Bpa_{3,13}$ cross-linked to calmodulin residues 55–84 plus 88–140.
†Two Met residues are modified in the cross-linking reaction. Control hydrolyses in which unmodified calmodulin fragments were analyzed showed normal values of Met.

TABLE IV. Amino Acid Analysis of the Trp_3–Bpa_{13} Photoadduct V8 Fragment

Amino acid	Expected*	Observed
Asx	6	5.4
Thr	3	2.9
Ser	1	0.9
Glx	4	3.8
Gly	3	3.0
Ala	2	2.0
Val	1	0.9
Met	3	1.6†
Ile	1	1.0
Leu	8	8.2
Phe	2	1.9
Lys	9	9.0
Arg	1	1.1
Pro	1	0.8

*Calculated for Trp_3–Bpa_{13} cross-linked to calmodulin residues 55–84.
†One Met residues is modified in the cross-linking reaction. Control hydrolyses in which unmodified calmodulin fragments were analyzed showed normal values of Met.

consisting of residues 55–84 of calmodulin plus the $Bpa_{3,13}$ peptide. Again, there was a gap in the se quence at position 17 corresponding to Met-71.

S. aureus V8 Digestion of Trp_3–Bpa_{13} Photoadduct

S. aureus V8 digestion of the Trp_3–Bpa_{13} photoadduct showed predominantly one new peak in the HPLC profile when compared with a calmodulin control (data not shown). The new peak eluted significantly later in the gradient than any other calmodulin V8 fragment (app. 36% acetonitrile) and was purified to homogeneity and identified by FAB/MS, amino acid analysis, and sequencing. The data are consistent with a fragment of calmodulin uniquely labeled at Met-71 by Trp_3–Bpa_{13}. Amino acid analysis for the fragment is shown in Table IV; the composition is consistent with that predicted for residues 55–84 of calmodulin plus Bpa–Trp. FAB/MS supported this sequence identification giving an $(M+H)^+$ of 5639.4 identical within experimental error to the calculated mass of 5638.7. Results for Edman sequencing of the peptide eluting in this peak are shown in Table V, and are consistent with the amino acid composition. For cycle 17, corresponding to residue 71 of calmodulin, no detectable amino acid was observed, suggesting that this is the position for covalent attachment of the Bpa residue from the peptide.

TABLE V. Amino Acid Sequence of Trp_3–Bpa_{13} Photoadduct V8 Fragment

Cycle	Amino acid (pmol)
1	Val (823)
2	Asp (630)
3	Ala (633)
4	Asp (521)
5	Gly (287)
6	Asn (279)
7	Gly (242)
8	Thr (466)
9	Ile (318)
10	Asp (373)
11	Phe (298)
12	Pro (144)
13	Glu (151)
14	Phe (117)
15	Leu (125)
16	Thr (150)
17	X
18	Met (115)
19	Ala (87)
20	Arg (48)
21	Lys (62)
22	Met (70)
23	Lys (36)
24	Asp (58)
25	Thr (59)
26	Asp (23)
27	Ser (18)
28	Glu (16)
29	Glu (26)
30	Glu (24)

Fluorescence Spectroscopy of the Trp_3–Bpa_{13} Photoadduct

Table VI gives a summary of the fluorescence emission maxima, anisotropy, and acrylamide quenching measurements for Trp_3 and Trp_3–Bpa_{13} in aqueous solution and in the calmodulin complex or photoadduct. As illustrated in Figure 4 the wavelength of maximum fluorescence emission for Trp_3–Bpa_{13} in aqueous solution is 345 nm; the corresponding value for the peptide:calmodulin photoadduct is observed at 321 nm, giving an overall blue shift of 24 nm.

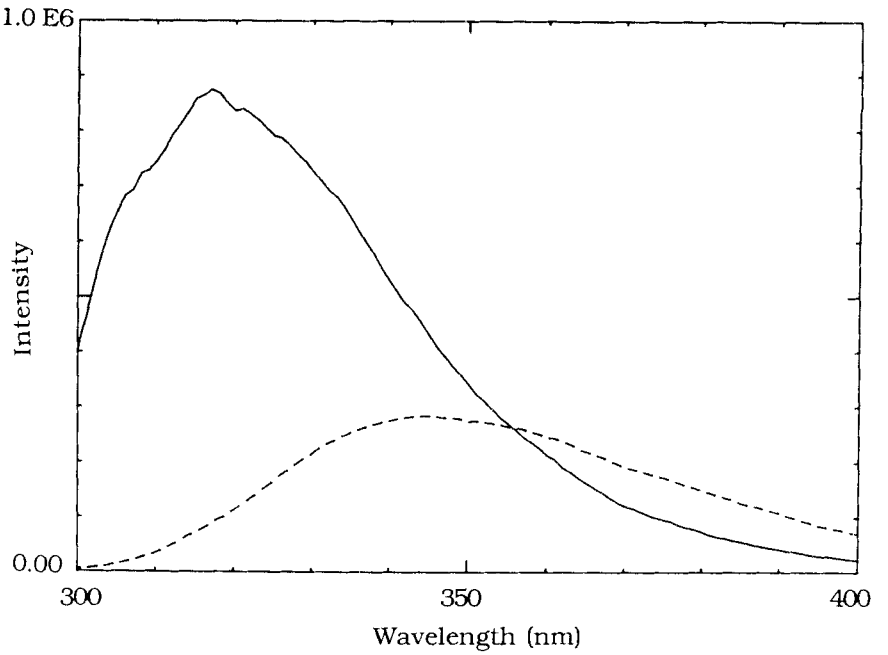

Fig. 4. Fluorescence emission spectra for Trp_3–Bpa_{13} (dotted) and the Trp_3–Bpa_{13}:calmodulin photoadduct (dashed). Spectra were measured for 10 μM peptide or photoadduct in 10 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 7.5 in a 1 cm rectangular cell on a Spex F222 fluorometer. Wavelength for excitation was 290 nm; slits were 2.7 nm for excitation and 3.6 nm for emission. All spectra were corrected for background fluorescence and the nonlinearity of the photomultiplier response.

TABLE VI. Fluorescence Parameters for Peptides and Peptide/Calmodulin Complexes

	λ_{max} (nm)	r_0	K_q (M^{-1})
Trp_3 alone	345	0.05	15.1
Trp_3–CaM complex	321	0.17	1.2
Trp_3–Bpa_{13} alone	345	0.07	n.d.
Trp_3–Bpa_{13} photoadduct	321	0.18	1.0

This large blue shift is consistent with a movement of the Trp ring from a polar solvent to a very nonpolar environment such as one might find in the interior of a protein. Similarly, the fluorescence maximum in Trp_3 undergoes a 24 nm blue shift when the peptide is bound to calmodulin.[13]

Fluorescence anisotropy measurements are sensitive to the rotational mobility of the indole ring. Trp_3–Bpa_{13} has an anisotropy of 0.07 in aqueous solution that increases to 0.18 when the peptide has been cross-linked to calmodulin. The large increase in anisotropy can be explained only by a large decrease in overall tumbling rate *as well as* a large degree of immobilization of the Trp residue in the complex.[12,13] For comparison, the Trp_3:calmodulin complex has an anisotropy of 0.17 while the anisotropy for the free peptide is 0.05.

In addition, the accessibility to acrylamide quenching of the Trp side chain in the Trp_3–Bpa_{13} complex is very low, having a particularly small Stern–Volmer quenching constant. Plots of F_0/F (where F and F_0 are the fluorescence intensities in the presence and absence of a given concentration of acrylamide, respectively) as a function of acrylamide concentration were linear for both peptides. The slope of F_0/F (K_q, the Stern–Volmer quenching constant) for Trp_3 in aqueous solution is 15.1 M^{-1} while K_q for the Trp_3:calmodulin complex is 1.2 M^{-1}. For the Trp_3–Bpa_{13} photoadduct, the Trp residue is even less accessible to acrylamide quenching as evidenced by a value for K_q of 1.0 M^{-1}.

The values for the emission maximum, anisotropy, and K_q were also measured for the noncovalent Trp_3–Bpa_{13}:calmodulin complex formed by mixing one equivalent of peptide and protein. HPLC analysis of the complex after the completion of the experiment indicated that photoadduct formation during the measurement of these parameters was negligible under our experimental conditions. The values observed for each parameter were identical to those determined for the covalent photoadduct, indicating that the covalent cross-link does not greatly affect the fluorescence results. The combined fluorescence results argue for an orientation of the peptide in the calmodulin–peptide complex where the Trp residue in the third position of the peptide is buried in a rigid, hydrophobic pocket on the surface of calmodulin.

DISCUSSION

The cross-linking and fluorescence results described above provide strong support for the hypoth-

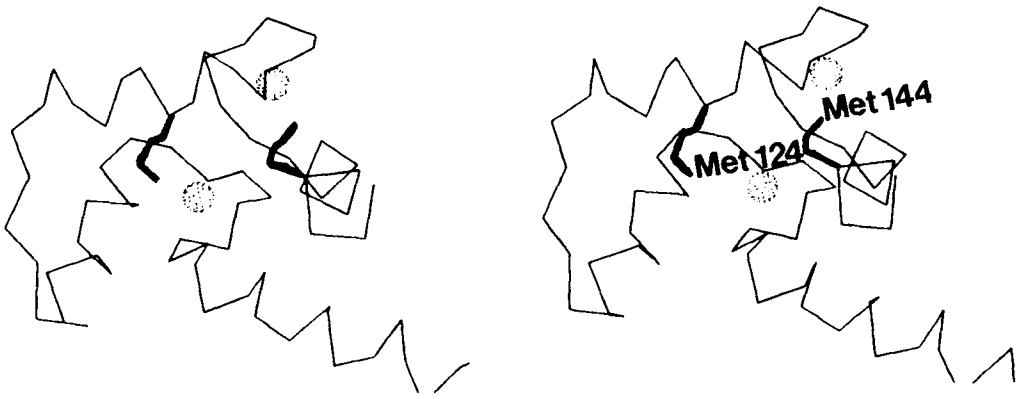

Fig. 5. Stereo diagram of the C_α backbone for the C-terminal lobe of calmodulin. Coordinates are from the 2.2 Å structure by Babu and co-workers.[4] The side chains for Met-124 and Met-144 are drawn in bold lines and calcium ions are depicted as van der Waals dot surfaces.

esis that the central helix of calmodulin functions as *a flexible tether* as illustrated in the model in Figure 1. We have previously identified the third position in a 17-residue calmodulin binding peptide to be buried in a hydrophobic pocket on the C-terminal lobe of calmodulin, while the thirteenth position of the model peptide is located within a somewhat less defined hydrophobic pocket on the N-terminal lobe.[11,13] Here we find that when the Bpa residue in the thirteenth position of Trp_3–Bpa_{13} is covalently cross-linked to the N-terminal hydrophobic patch, the fluorescence properties of the Trp residue in the third position are modulated in a way analogous to those observed for the Trp_3:calmodulin complex. This result provides strong evidence for a complex in which the peptide simultaneously binds to both lobes of calmodulin.

Additional evidence for a flexible central helix in calmodulin is shown in our results with the $Bpa_{3,13}$ photoadduct, which labels Met-71 on the N-terminal lobe and Met-124 on the C-terminal lobe. It is interesting to compare these results with those obtained for the Bpa_3, Trp_3–Bpa_{13}, and Bpa_{13} peptides. The Trp_3–Bpa_{13} and Bpa_{13} peptides both label Met-71, suggesting that the Bpa in the thirteenth position of $Bpa_{3,13}$ is the one that cross-links to Met-71. It follows that the Bpa in position 3 labels residue Met-124. Interestingly, a different residue on the C-terminal lobe of calmodulin, Met-144, is labeled by the Bpa_3 peptide. Met-124 and Met-144 are both located on the C-terminal lobe of calmodulin (Fig. 5) where they line opposite sides of the hydrophobic cavity into which the Bpa residue is believed to bind.[11] Modeling studies indicate that either Met-124 or Met-144 may be labeled, depending on the orientation of the Bpa residue in the hydrophobic cavity.

The results described for the Trp_3–Bpa_{13} and $Bpa_{3,13}$ photoadducts are consistent with a peptide bound conformation for calmodulin in which Met-71 and Met-124 are significantly closer together than in the crystal structure of the protein. By introduction of a kink in the central helix linking the two domains of the protein as described by Persechini and Kretsinger,[3] a conformation can be obtained for calmodulin that is consistent with both the labeling and the fluorescence studies described herein. Figure 6 illustrates a model for the peptide bound conformation of calmodulin. Using the calmodulin coordinates predicted by Persechini and Kretsinger, a 17-residue peptide has been positioned such that Trp residues in both the third and thirteenth positions can fit into the hydrophobic clefts in the C-terminal and N-terminal lobes of calmodulin, respectively. This orientation of the peptide helix, however, is opposite that predicted by Persechini and Kretsinger, who suggested that the N-terminus of an MLCK peptide helix would be oriented toward the N-terminal domain. It is possible that different peptides will bind with orientations other than that defined for the set of peptides described herein. These results suggest that the helix between the two domains of calmodulin is sufficiently flexible to bring the two domains close enough together to form a contiguous site for peptide or enzyme binding. The mechanism by which calmodulin is able to specifically recognize any one enzyme may then be governed by the orientation of the lobes with respect to one another.

Calmodulin's ability to recognize and interact with such a wide diversity of proteins is probably thus governed by the inherent flexibility in the linker sequence between the two domains. This explains why so many peptides and proteins compete for binding to calmodulin. All most likely interact at

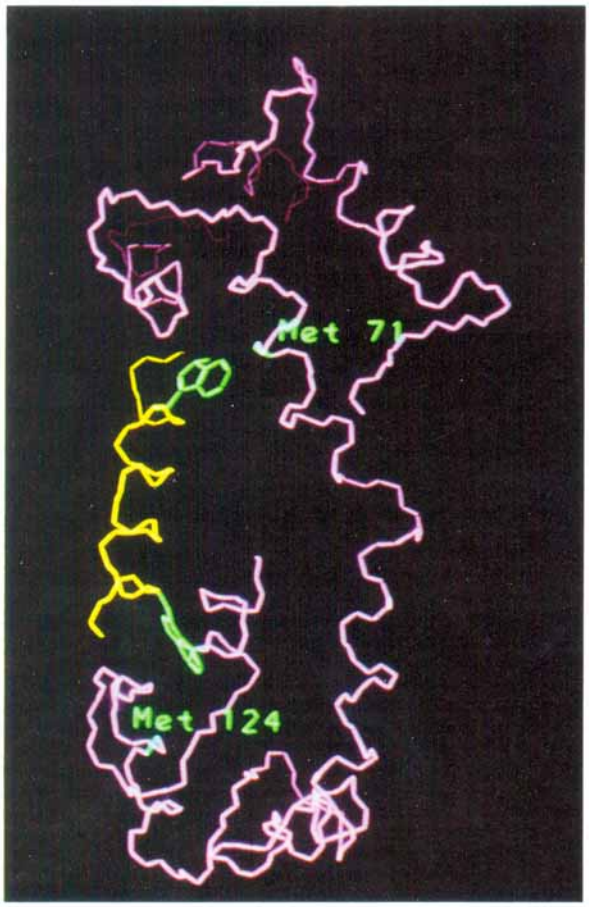

Fig. 6. Computer graphics representation of a calmodulin: peptide complex. Calmodulin backbone atoms are shown in purple with the calmodulin residues cross-linked by $Bpa_{3,13}$ (i.e., Met-71 and Met-124) highlighted in green; coordinates for the model are from the structure described by Persechini and Kretsinger.[3] The peptide molecule with a tryptophan residue in both the third and thirteenth position is shown in yellow. By introduction of a bend in the central helix of calmodulin, the two domains can be oriented such that they cooperatively form a peptide-binding site. In this orientation, the tryptophan residues in the third and thirteenth positions of the peptide are buried in the hydrophobic clefts on the C-terminal and N-terminal domains of calmodulin, respectively.

a similar, overlapping site but by appropriate adjustment of the orientation of the two domains with respect to one another calmodulin can specifically and uniquely activate each enzyme.

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