Photolabeling of Calmodulin with Basic, Amphiphilic α-Helical Peptides Containing p-Benzoylephonylalanine*

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A novel photoreactive amino acid has been incorporated synthetically into two model peptides and the calmodulin-binding domain from myosin light chain kinase. Cross-linked photoproducts of each peptide with calmodulin have been prepared and digested by chemical and/or enzymatic methods to determine the site of label attachment. Depending on the position of the photoprobe in the peptide sequence, either Met-144 or Met-71 is photolabeled. These results are discussed in relation to the three-dimensional structure of calmodulin obtained crystallographically and the known solution properties of calmodulin.

Calmodulin is involved in the calcium-dependent regulation of a broad spectrum of enzymes including protein kinases, adenylate cyclases, and phosphodiesterases (1). The mechanism by which calmodulin activates or modulates such a wide variety of enzymes is becoming increasingly clear through the use of model systems (2, 3). By using synthetic, model peptides it has been shown that calmodulin is capable of binding basic, amphiphilic α-helices with extremely high affinity ($K_{d}$ < 10 nM, Refs. 4 and 5). This structural feature is also found in the putative calmodulin-binding domains of numerous target enzymes including myosin light chain kinases (MLC kinases, Refs. 5–11). Ca$^{2+}$-calmodulin-dependent protein kinase II (12, 13), phosphofructokinase (14), the calmodulin-binding domain of the human erythrocyte calcium pump (15), phospholysine b kinase (16), and lens gap junction protein (17). Peptides corresponding to the putative calmodulin-binding domains of many of these target enzymes have been shown to bind tightly to calmodulin (6, 8, 9, 13–17), and circular dichroism spectroscopy of the complexes suggests that they bind in helical conformations (6, 9, 16, 17). Surprisingly, there is relatively little precise sequence conservation common to the entire set of peptides. Therefore, we suggested that calmodulin recognizes a structural feature common to all of these peptides (4).

To help better define how calmodulin binds amphiphilic peptides we have prepared photoreactive derivatives of our previously described model calmodulin-binding peptide, Trp-3 (Table 1), and of the putative calmodulin-binding domain of smooth muscle myosin light chain kinase (6). The photolabel, p-benzoylephonylalanine (Bpa, Refs. 19 and 20) was chosen because of its clean photochemistry and high stability to the conditions of peptide synthesis. In contrast to most other photoaffinity labels that generate highly reactive electrophilic species (25), benzophenone derivatives react via a triplet biradical mechanism (Scheme I) with a high preference for reaction with C–H bonds (19–24). Thus, the Bpa residue should be particularly useful for labeling calmodulin, whose peptide-binding site or sites are believed to contain a number of apolar side chains (2, 3, 27).

![Scheme I](image)

**Scheme I**

The fluorescence properties of a set of peptides in which tryptophan was placed at each possible location in a 17-residue sequence have been used to illustrate the helical binding conformation of the peptide and to identify peptide residues in close contact with the protein surface (28). These studies revealed that a tryptophan side chain at position 3, 7, 10, 13, or 16 of the peptide was partially or completely buried in an apolar environment at the calmodulin-peptide interface; calmodulin binding measurements indicated that a tryptophan side chain could be accommodated at these positions without decreasing the affinity. The side chains of tryptophan and Bpa are similar in size and hydrophobicity so positions where a tryptophan could be accommodated were prime candidates for replacement with Bpa. Two Bpa-containing peptides, Bpa-3 and Bpa-13 were prepared (Table 1). In addition, we prepared a peptide (MLC kinase-Bpa) corresponding to the putative calmodulin-binding domain of MLC kinase with a Bpa substitution for the tryptophan at position 4. The position of the Bpa residue in this peptide is homologous to the position of the Bpa in the Bpa-3 peptide (Table I). Upon irradiation with 350 nm light, complexes of these peptides with calmodulin react in excellent yield, and the positions of cross-linking can be easily determined by Edman sequence analysis and mass spectrometry. Bpa-3 and MLC kinase-Bpa react with Met-144 of calmodulin, indicating that the two peptides are bound similarly. When the Bpa residue was substituted at position 13 of the peptide, Met-71 of calmodulin was cross-linked. These results are discussed in relation to the known structural properties of calmodulin.
EXPERIMENTAL PROCEDURES

Materials—Calmodulin (bovine brain) was obtained from Pharmacia LKB Biotechnology Inc. or Calbiochem. Staphylococcus aureus V8 protease was from ICN, and cyanogen bromide and trypsin were from Sigma. All were used as received with no further purification. Fmoc-L-Val was the generous gift of J. C. Kauer (Du Pont). [4H] Acetic anhydride was purchased from Du Pont-New England Nuclear. Fmoc- and Boc-protected amino acids and amino acid resins were purchased from NovaBiochem (Lauffenberg, Switzerland), Bachem (Torrence, CA), or Advanced ChemTech (Louisville, KY).

Synthesis of Fmoc Protected Gly-Merrifield Resin—Acetic anhydride was used in the dimethylformamide solution of the peptide, and the reaction was continued until the resin was free of solvents, as determined by the coupling test (29). After completion of the synthesis, and removal of the N-terminal Fmoc group, the peptides were acetylated by reaction with a 1 fold excess of acetic anhydride and a 2-fold excess of trifluoroacetic acid for 3 h. The coupling was determined complete by the ninhydrin test (29).

Purification of Fmoc-Bpa-3 and Bpa-13—Bpa-3 and Bpa-13 were synthesized from Fmoc-Gly resin using the RAMPs method (Technical Bulletin, Du Pont-New England Nuclear). The Bpa residue was incorporated using a 2-fold excess of the corresponding Fmoc-protected hydroxybenzotriazole ester prepared by reaction of 2 eq of Fmoc-L-Bpa with 2 eq of hydroxybenzotriazole in dimethylformamide for 5 min at room temperature. The reaction was then heated at 80 °C for 3 h, and the coupling was determined complete by the ninhydrin test (29). After completion of the synthesis, and removal of the N-terminal Fmoc group, the peptides were acetylated by reaction with a 1 fold excess of acetic anhydride and a 2-fold excess of trifluoroacetic acid for 24 h. Following acetylation, Bpa-3 and Bpa-13 were cleaved from the resin with 90% trifluoroacetic acid, 10% thioanisole for 6 h at room temperature. Both crude peptides were purified in a single step by reverse phase HPLC using a Hamilton PRP-1 semi-preparative column (purchased from Pierce) and a gradient of 43-58% aqueous acetonitrile containing 1% trifluoroacetic acid, at 0.75%/min and a flow rate of 4.0 ml/min. Fractions containing pure peptide were combined and lyophilized.

RESULTS

Photolabeling of Calmodulin—The Bpa-3-cammodulin photoaduct was purified from the reaction mixtures by reverse phase HPLC using a semi-preparative Vydac C18 column. Fractions containing photoaduct were lyophilized, dissolved in a small amount of 70% formic acid, and CNBr-digested to a ratio of 10:1 (w/w) CNBr/calmodulin. After 24 h digestion at room temperature, samples were lyophilized and stored dry at −20 °C until purification. Purification of tritium-labeled fragments was achieved in the same manner as described above for the enzymatic digests. Fragments were identified by N-terminal sequencing and amino acid analysis.

Miscellaneous—Amino acid analyses were obtained using a Beckman Amino Acid Analyzer 119 CI ion exchange system with ninhydrin detection. N-terminal sequencing analyses were obtained on Applied Biosystems 470A Protein/Peptide Sequencer interfaced with an Applied Biosystems 120A PTH analyzer. Fast atom bombardment/mass spectrometry data were collected using a VG ZAB-E mass spectrometer fitted with an Ion Tech gun using xenon as the ionizing gas. Coordinates for calmodulin were generously supplied by Dr. Bogg (University of Alabama, Birmingham) and are now available from the Brookhaven Protein Data Bank (1986) (41).

Photolabeling—Photoreactions were carried out in polystyrene dishes (Costar 24-well dishes) with the light source (Bilux Ray Lamp, Long Wave UV-366 WM, UVF, INC, San Gabriel, CA) positioned 1-2 cm over the wells. Reactions were carried out at 0.5 or 1.0 mg/ml calmodulin with a peptide/calmodulin ratio of 0.9:1.0 in 0.25 mM ammonium acetate, pH 8.0, containing 10 mM dithiothreitol and 3 mM CaCl2. Photoaducts of calmodulin with Bpa-3 or MLC kinase-Bpa used for cyanogen bromide digestion were prepared as described previously.

Enzymatic Digestions—S. aureus V8 digestion of photoaducts were carried out in photolysis reaction buffer after addition of urea to a final concentration of 4 M, EDTA to a final concentration of 10 mM, and adjustment of pH to 7.8. Dry protease was added to a final protease:calmodulin molar ratio of approximately 1:10. Reactions were most successful when allowed to proceed overnight at 37 °C. For each complex a calmodulin standard was digested simultaneously for comparison. Tryptic digestions were performed in 25 mM ammonium bicarbonate, pH 8.4, at 37 °C overnight. The enzyme to peptide molar ratio was approximately 1:10. Purification of V8 proteolytic fragments was achieved by reversed phase HPLC using a Vydac C18 analytical column. In most cases a slow gradient (0.5%/min) of aqueous acetonitrile (90%) acetonitrile, 0.1% trifluoroacetic acid) yielded the required separation. All peaks that appeared in a photoaduct digestion but not a calmodulin control were collected and counted for tritium. Tritium-labeled fragments were identified by amino acid analysis, FAB/MS, and N-terminal sequencing.

Cyagen Bromide Digestions—The Bpa-3-calmodulin photoaduct was purified from the reaction mixtures by reverse phase HPLC using a semi-preparative Vydac C18 column. Fractions containing photoaduct were lyophilized, dissolved in a small amount of 70% formic acid, and CNBr-digested to a ratio of 10:1 (w/w) CNBr/calmodulin. After 24 h digestion at room temperature, samples were lyophilized and stored dry at −20 °C until purification. Purification of tritium-labeled fragments was achieved in the same manner as described above for the enzymatic digests. Fragments were identified by N-terminal sequencing and amino acid analysis.

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Photolabeling of Calmodulin

Enzymatic Digestion of Bpa-13-calmodulin Photoadduct

Examination of the S. aureus V8 cleavage products of the Bpa-13-calmodulin photoadduct in Fig. 4 reveals the presence of four tritium-labeled peaks in the photoadduct digest profile. As indicated in Table IV, the amino acid sequence of these four fragments corresponds to the same fragment of calmodulin: residues 55–84. Cycle 17, corresponding to residue 71 in the calmodulin sequence, indicated very low values for methionine; normal values for residue 72 and each subsequent residue were observed. The low levels of methionine observed for cycle 17 in peaks A and B probably reflect a small amount of decomposition of the photoadduct that occurs during storage of the samples or sequencing. FAB/MS yielded an average (M + H)⁺ of 5566 equal to the calculated mass for fragment 55–84 of calmodulin plus Bpa-13. This assignment was confirmed by further digesting the fragment with trypsin, which gave an (M + H)⁺ of 2839. Trypsin cleavage of the photoadduct after Arg-75 of calmodulin, and lysines 12 and 15 of Bpa-13 (yielding residues 55–75 with Bpa-Leu-Lys attached) was identified by FAB/MS with (M + H)⁺ = 2839.

It should also be noted that peak A contained a second sequence, corresponding to residues 141–148 (Table IV). As was the case for the Bpa-3/calmodulin photoadduct, the relative yields of the phenylthiohydantoin derivatives for the sequences in peak A, it can be estimated that Met-71 was labeled with at least a 10-fold preference over Met-144 for the Bpa-13 peptide.

Enzymatic Digestion of the MLC Kinase-Bpa Photoadduct

Comparison of the reverse phase HPLC profile for the S. aureus V8 digest of the MLC kinase-Bpa/calmodulin adduct versus the calmodulin control in Fig. 5 shows three new peaks in the photoadduct profile that contain the tritium label. Sequencing of these fragments (Table V) indicated that each of these peaks contained the same calmodulin fragment, namely residues 141–148 of the calmodulin sequence. In all cases the fourth cycle of sequencing shows very low levels of the methionine, normally found at position 144; subsequent

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**Fig. 1. Time course for photolabeling of calmodulin with MLC kinase peptide.** The reaction contained calmodulin (CaM) and MLC kinase peptide at a ratio of 1.0:9.9, 0.25 mM ammonium acetate, pH 8.0, 3 mM CaCl₂, and 10 mM dithiothreitol. The concentration of calmodulin was 1 mg/ml. Aliquots were removed at t = 0, 30 min, and 2.25 h for HPLC. HPLC separation was achieved using a Vydac analytical C18 column, flow rate 1 ml/min, and an aqueous acetonitrile gradient as illustrated in the top panel.

Acid analysis indicated that the second peak was the starting peptide, Bpa-3, while the composition of the first peak correlates with the composition for the CNBr fragment of calmodulin including residues 125–148 plus Bpa-3 (Table II). The free, starting Bpa-3 peptide is not due to an incomplete reaction during the photolysis step, as could be shown in experiments in which the photoadduct was purified by HPLC prior to digestion, but rather it appears that the photoadduct is only partially stable under the conditions of CNBr digestion. This problem was even more pronounced for the Bpa-13 analogue, so we turned to enzymatic methods of digestion for further analysis of the photoadducts.

**Enzymatic Digestion of Bpa-3 Photoadduct—The S. aureus V8 proteolytic digestion of the calmodulin-Bpa-3 photoadduct and the corresponding calmodulin control are shown in Fig. 3. Two unique peaks containing significant tritium radioactivity (peaks A and B) were observed in the photoadduct, but not the control. Sequence analysis was used to identify the calmodulin fragment since the N terminus of the Bpa-3 peptide was acetylated, and there are no V8 digestion sites in its sequence. As shown in Table III, Edman sequencing identified the labeled fragment as the C-terminal V8 fragment of calmodulin, corresponding to residues 141–148 (31). Both of these peaks yielded the same sequence and mass spectral results, indicating that two isomers were created at the photolysis step. These isomers most probably arise from different atomic positions of insertion of the Bpa moiety into the methionine side chain. The methionine at position 4 (corresponding to residue 144 of native calmodulin) was not observed, suggesting that this was the point of covalent cross-linking. No unusual phenylthiohydantoin-derivative peak (e.g., oxidized methionine derivatives) was observed at this position, nor was significant radioactivity observed in the washes during this step, as would be expected if the missing methionine remained covalently attached to the Bpa-3 peptide (which should remain firmly absorbed to the filter). Values for methionine in cycle five, corresponding to residue 145 of calmodulin returned to normal levels. Fast atom bombardment mass spectrometry (FAB/MS) supported the sequence identification yielding an (M + H)⁺ of 3126 for peak A and an (M + H)⁺ of 3127 for peak B, which were identical within our experimental error to the calculated mass of 3126 (+ H⁺) for the calmodulin fragment including residues 141–148: Phe-Val-Gln-Met-Met-Thr-Ala-Lys plus the Bpa-3 peptide.
**Photolabeling of Calmodulin**

**FIG. 2.** CNBr cleavage products of calmodulin (A) and the Bpa-3-calmodulin photoadduct (B) and a calmodulin control. Fractions were collected at 1-min intervals and counted for $^3$H to identify peptide containing fragments. The gradient illustrated in this figure and Figs. 3–5 corresponds to the aqueous acetonitrile solvent system described under "Experimental Procedures."

**TABLE II**

Amino acid analysis of peak 1

| Residue | Expected | Observed |
|---------|----------|----------|
| Asx     | 4.0      | 4.0      |
| Thr     | 1.0      | 1.3      |
| Ser     | 0        | 0.3      |
| Glx     | 5.0      | 5.3      |
| Gly     | 2.0      | 2.0      |
| Ala     | 2.0      | 2.1      |
| Val     | 2.0      | 1.8      |
| Ile     | 2.0      | 1.8      |
| Leu     | 8.0      | 8.0      |
| Tyr     | 1.0      | 0.9      |
| Phe     | 1.0      | 1.2      |
| Lys     | 8.0      | 8.1      |
| His     | 0        | 0        |
| Arg     | 1.0      | 1.3      |
| Pro     | 0        | 0        |

**FIG. 3.** *S. aureus* V8 protease cleavage products of Bpa-3-photoadduct (A) or calmodulin control (B). Fractions were collected at 0.5-min intervals and counted for $^3$H to identify fragments containing significant radioactivity.

**TABLE III**

N-terminal sequence of Bpa-3 photoadduct V8 digest

| Cycle | Peak A$^*$ | Peak B$^*$ | CaM$^+$ (141–148) |
|-------|------------|------------|-------------------|
| 1     | Phe (202)  | Phe (132)  | Phe (1453)        |
| 2     | Val (157)  | Val (103)  | Val (1007)        |
| 3     | Gln (132)  | Gln (36)   | Gln (855)         |
| 4     | X          | X          | Met (1143)        |
| 5     | Met (154)  | Met (17)   | Met (1334)        |
| 6     |             |            | Thr (535)         |
| 7     | Ala (707)  |            |                   |
| 8     | Lys (244)  |            |                   |

$^*$ Only five cycles of sequencing were performed on these samples.

$^+$ CaM, calmodulin.

**FIG. 4.** *S. aureus* V8 proteolytic digestion of BPA-13 photoadduct. Significant radioactivity was discovered in peaks A–D by counting fractions collected at 0.5-min intervals for $^3$H. Cycles correlate with the expected sequence for residues 145–148. FAB/MS analysis $(M + H)^+ = 3356.7$ (calculated $(M + H)^+ = 3356$) supported the assignment of this peak to frag-
TABLE IV  
N-terminal sequence of Bpa-13 photoadduct V8 digest

| Cycle | Peak A | Peak B | Peak C | Peak D | CaM\(^*\) (55–77) |
|-------|--------|--------|--------|--------|------------------|
| 1     | Val/Asp (305/534) | Val (449) | Val (1032) | Val (324) | Val (1427) |
| 2     | Asp/Val (348/329) | Asp (509) | Asp (779) | Asp (779) | Asp (1102) |
| 3     | Ala/Gln (357/269) | Ala (542) | Ala (504) | Ala (504) | Ala (1290) |
| 4     | Asp/Met (248/35) | Asp (477) | Asp (821) | Asp (821) | Asp (903) |
| 5     | Gly/Met (259/302) | Gly (328) | Gly (358) | Gly (275) | Gly (977) |
| 6     | Asn/Thr (153/125) | Asn (345) | Asn (145) | Asn (145) | Asn (843) |
| 7     | Gly/Ala (185/223) | Gly (267) | Gly (245) | Gly (164) | Gly (837) |
| 8     | Thr/Lys (89/89) | Thr (136) | Thr (248) | Thr (156) | Thr (573) |
| 9     | Ile (125) | Ile (139) | Ile (242) | Ile (110) | Ile (652) |
| 10    | Asp (98) | Asp (171) | Asp (310) | Asp (132) | Asp (475) |
| 11    | Phe (144) | Phe (135) | Phe (358) | Phe (127) | Phe (601) |
| 12    | Pro (84) | Pro (84) | Pro (215) | Pro (82) | Pro (512) |
| 13    | Glu (63) | Glu (109) | Glu (219) | Glu (132) | Glu (144) |
| 14    | Phe (81) | Phe (94) | Phe (203) | Phe (77) | Phe (1116)\(^*\) |
| 15    | Leu (64) | Leu (80) | Leu (136) | Leu (82) | Leu (987) |
| 16    | Thr (36) | Thr (50) | Thr (100) | Thr (68) | Thr (708) |
| 17    | Met (11) | Met (14) | X | X | Met (1058) |
| 18    | Met (59) | Met (85) | Met (129) | Met (100) | Met (1091) |
| 19    | Ala (48) | Ala (65) | Ala (107) | Ala (64) | Ala (996) |
| 20    | Arg (18) | Arg (39) | Arg (77) | Arg (35) | Arg (205) |
| 21    | Lys (39) | Lys (54) | Lys (88) | Lys (50) | Lys (640) |
| 22    | Met (47) | Met\(^*\) | Met (119) | Met (78) | Met (891) |
| 23    | Lys (41) | Lys\(^*\) | Lys (102) | Lys (50) | Lys (258) |
| 24    | Asp (27) | Asp\(^*\) | Asp (70) | Asp (71) | Asp (475) |
| 25    | Thr (19) | Thr\(^*\) | Thr (43) | Thr (51) | Thr (573) |
| 26    | Asp (32) | Asp (49) | Asp (85) | Asp (71) | Asp (707) |
| 27    | Ser (8) | Ser (10) | Ser (14) | Ser (15) | Ser (284) |
| 28    | Glu (16) | Glu (23) | Glu (57) | Glu (84) | Glu (82) |
| 29    | Glu (21) | Glu (30) | Glu (55) | Glu (82) | Glu (82) |
| 30    | Glu (19) | Glu (26) | Glu (42) | Glu (73) | Glu (73) |

\(^*\) CaM, calmodulin.

\(^*\) Calmodulin is normally cleaved after Glu-67 in the presence of S. aureus V8 protease. The apparently anomalously high values for Phe in cycle 14 are simply due to two fragments being sequenced to obtain the full sequence from residue 55–77 of calmodulin.

\(^*\) Computer outage: no picomole values for amino acids were obtained; identification of amino acids was made by inspection of the HPLC profiles for these cycles.

Fig. 5. S. aureus V8 proteolytic digestion of MLC kinase photoadduct or calmodulin control. Peaks A, B, and C were identified as peptide labeled by \(^3\)H counting of fractions collected at 0.5-min intervals.

TABLE V  
N-terminal sequence of MLC kinase photoadduct V8 digest

| Cycle | Peak A | Peak B | Peak C | CaM\(^*\) (141–148) |
|-------|--------|--------|--------|------------------|
| 1     | Phe (752) | Phe (300) | Phe (731) | Phe (1453) |
| 2     | Val (606) | Val (250) | Val (587) | Val (1007) |
| 3     | Gln (405) | Gln (203) | Gln (434) | Gln (855) |
| 4     | Met (46) | Met (89) | Met (67) | Met (1143) |
| 5     | Met (506) | Met (183) | Met (633) | Met (1334) |
| 6     | Thr (163) | Thr (79) | Thr (227) | Thr (135) |
| 7     | Ala (330) | Ala (174) | Ala (488) | Ala (707) |
| 8     | Lys (149) | Lys (75) | Lys (210) | Lys (244) |

\(^*\) CaM, calmodulin.

with large, aromatic substituents at either positions 3 or 13 bind to calmodulin competitively with respect to one another, the photolysis of Bpa-13 was repeated in the presence of the calmodulin-binding peptide, Trp-3. 20 \(\mu\)M calmodulin plus 18 \(\mu\)M Bpa-13 were photolyzed as described above in the presence or absence of a 5-fold excess of Trp-3. Analysis of the products by HPLC or sodium dodecyl sulfate gel electrophoresis (data not shown) indicated that the addition of the Trp-3 peptide decreased the level of photolabeling to less than 5% that observed in the absence of Trp-3, indicating that this peptide bound more tightly than Bpa-13 to calmodulin. Previously, we showed that substitution of a tryptophan for leucine at the 3rd position of the model peptide resulted in a 10-fold increase in affinity for calmodulin, whereas at position 13 it had little effect on the affinity (28).

**DISCUSSION**

The results described herein demonstrate the usefulness of the Bpa residue as a photoaffinity probe that reacts cleanly

**Competition between Trp-3 and Bpa-13**

Previously we have described the binding competition between Trp-3 and Bpa-3 (20). To determine whether peptides...
and in high yield with proteins. The amino acid is extremely stable to the conditions of peptide synthesis and can be incorporated into peptides using either Boc or Fmoc protecting schemes. The atomic positions on a protein most likely to react with the triplet state of the Bpa residue may be inferred from the mechanism of insertion (Scheme 1), which involves hydrogen abstraction in the first and rate limiting step (21). Particularly reactive C–H bonds include tertiary centers (e.g. the C=C–H bond of leucine or the C=C–H bond of valine) and bonds adjacent to heteroatoms (C=C–H bonds, or bonds adjacent to the thioether of methionine). In the case of methionine it is possible that the reaction may occur through the intermediacy of a radical anion/cation pair (21) involving the thioether, which may account for the high reactivity of Met-71 and Met-144. In addition, computer-aided model building illustrates a large increase in solvent accessibility for 4 methionines in each of the hydrophobic patches upon calcium binding to calmodulin (32). Since the model peptides bind calmodulin in a calcium-dependent manner (i.e. when these methionine residues are exposed) this may account for the observation that in these experiments only methionine residues are labeled. The specificity of labeling of Met-71 and Met-144 is illustrated, however, by the observation that the methionine residues immediately adjacent to 71 and 144 (i.e. 72 and 145) are not labeled at all. Recently, the Bpa residue has also been used to label cAMP-dependent protein kinase (26). A peptide inhibitor of the enzyme incorporating this amino acid was found to photolabel a glycine and a methionine residue, previously localized to this enzyme’s active site. In this work as well as that reported here, the precise atomic positions of attachment were not determined. Our observation that digestion of the radiolabeled photoadducts gives multiple separable radioactive peptides with identical amino acid sequences and mass spectra would indicate that there are multiple atomic positions of attachment and/or that multiple stereoisomers are formed.

The crystal structure of calmodulin (33, 34) shows two, homologous domains connected by a long, central helix (Fig. 6). The C-terminal domain contains an exceedingly hydrophobic cavity large enough to accommodate the Bpa or other bulky, hydrophobic residue (Fig. 7). Consistent with this structure when the Bpa residue occupied the third position of the model peptide or MLC kinase peptide, the sole residue on calmodulin that was modified was Met-144 which is located in this cavity. This might account for the fact that the substitution of a tryptophan at the third position of our model peptide results in a 10-fold increase in its affinity for calmodulin. A tryptophan residue often occupies a homologous position in natural peptides such as the MLC kinase peptide described here (Table I, Ref. 2).

The Bpa-13 peptide labels Met-71 and Met-144 in about a 10:1 ratio, indicating that it has two possible binding orientations although the orientation placing the Bpa residue toward the N-terminal lobe is substantially preferred. Met-71 is homologous to Met-144 in the amino acid sequence of calmodulin (31) and is a part of a hydrophobic cavity similar to that of the C-terminal domain although the cavity appears to be less well defined than on the C-terminal domain. As noted earlier, both Met-71 and Met-144 have been predicted to experience a large increase in their solvent accessibilities when apo-calmodulin binds four Ca^{2+} ions (32), consistent with our observation that these peptides bind to and specifically label calmodulin only in the presence of excess Ca^{2+} (20).

As illustrated in Fig. 6, the primary sites of insertion for the Bpa-3 and Bpa-13 peptides are about 30 Å distant in space (C=C–C), although the two photoprobes are separated by only about three helical turns (about 15 Å) in the calmodulin-binding peptide. Even if the peptide were bound in a totally extended conformation, it does not appear to be sterically reasonable to bridge the two sites because of unfavorable

\[ \text{FIG. 7. Computer graphics illustration of the region surrounding Met-144. Calmodulin coordinates are from the 2.2 Å structure described by Babu and co-workers (42). The molecular surface illustrated here was generated using the Connolly algorithm (43) and has been color-coded as follows: hydrophobic residues green, acidic residues red, basic residues blue, neutral polar residues and glycine white. The tryptophan residue docked in the hydrophobic cleft has been colored yellow. Met-144 is located at the bottom right of the figure with its side chain extending up into the hydrophobic pocket in the C-terminal domain.} \]

\[ \text{FIG. 6. Ball and stick diagram of calmodulin. For comparison, a 17-residue helical peptide is shown next to the calmodulin structure. Only Cα carbons are shown, and the residues photolabeled (residues 71 and 144 of calmodulin, 3 and 13 of the peptide) are indicated as filled circles. The calmodulin structure is redrawn from Ref. 35.} \]

\[ \text{2 K. T. O’Neil, unpublished results.} \]
contacts with the protein. The most trivial explanation for this observation would be that calmodulin contains two possible peptide-binding sites (one on each domain) that can bind these peptides, and the position of the Bpa determines which site will have the highest affinity. In fact, we have found that calmodulin has one very high affinity binding site for model, amphiphilic peptides, but at high peptide and protein concentrations secondary binding sites can be detected (36). Therefore, the labeling studies were performed with calmodulin in slight molar excess over the peptide.

A more interesting explanation of our data would be that in the complex with amphiphilic peptides, the two domains actually are much closer than in the crystal structure and they make a single peptide-binding site. This is consistent with our finding that the Trp-3 peptide binds competitively with respect to Bpa-13 to calmodulin. Furthermore, an increasing body of experimental evidence provides strong support to suggest that the two domains are closer together in solution than in the crystal structure. 1) Low angle x-ray scattering studies of calmodulin in solution are consistent with the two domains having about the same radii as in the crystal structure, but being, on the average, about 5-10 Å closer together (37). Whether this represents a static "bend" in the central helix, or a dynamically interconverting ensemble of conformations of the central, connecting sequence could not be determined by this technique. 2) Mutants of calmodulin with one to four amino acids deleted from the central helix observed in the crystal structure, are all able to activate MLC kinase and NAD kinase with very similar activation parameters (38). 3) Mutants in which the two domains have been purposefully held closer together than in the crystal structure by introduction of cross-links into specific locations are highly active toward activation of target enzymes (39). 4) NMR studies have shown that changes occur in the chemical shifts and calcium-binding properties in both domains when amphiphilic peptides bind to calmodulin (9, 40). While any one of these experiments have a number of possible interpretations, collectively they argue that the central helix functions as a flexible tether allowing the individual domains of calmodulin to fold over to make a single, continuous peptide-binding site.

To address the question of multiple binding sites versus a change in the conformation of calmodulin when peptide is bound, we have synthesized the peptide containing Bpa in both the 3rd and the 13th position. Bpa-3-13 forms one homogeneous adduct with calmodulin. This adduct is extremely stable against proteolytic digestion consistent with the presence of a very well packed complex. Work is currently in progress to characterize this photoadduct and identify the site(s) of covalent labeling.

By adjusting the conformation of the intervening sequence, the two domains should be able to adopt a variety of mutual orientations, allowing the protein to bind a variety of different peptides. Also contributing to calmodulin's broad specificity is the fact that the exposed hydrophobic surfaces on its individual domains are largely made up of methionine residues. Of the hydrophobic side chains, methionine has the most possible conformers, and hence might be expected to make minor adjustments to accommodate variations in the structures of the bound peptides. Thus, by taking advantage of its inherent flexibility, calmodulin can bind a variety of amphiphilic peptides in a fundamentally similar manner. This explains why various peptides compete for one another for binding to calmodulin. On the other hand, although the specific interaction sites overlap from one peptide to another, they are nonidentical. Therefore, modifications in calmodulin's structure or changes in solution conditions should have differential effects on the properties of various peptide/protein complexes. This conclusion should also hold for calmodulin's interactions with target enzymes, although the larger size of target enzymes allows for interactions that extend over larger and more diverse surfaces than in the case of peptides.

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