Photoaffinity Labeling Study of the Interaction of Calmodulin with the Plasma Membrane Ca^{2+} Pump†

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ABSTRACT: Bovine brain calmodulin was labeled with synthetic peptides corresponding to the calmodulin-binding domain of the erythrocyte plasma membrane Ca^{2+}-ATPase. One 20-amino acid peptide and two 28-amino acid peptides were used, carrying L-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanine residues in position 9 (peptides C20W* and C28W*) and position 25 (peptide C28WC*), respectively. The localization of the contact regions between calmodulin and the N- and C-terminal portions of the peptides was the aim of this study. The three peptides were N-terminally blocked with a ^3H-labeled acetyl group to facilitate the identification of labeled fragments after isolation and digestion. The binding site for phenylalanine 25 was identified in the N-terminal domain of calmodulin while the phenylalanine derivative in position 9 labeled the C-terminal domain. Fluorescence studies using the dansylated N- and C-terminal halves of calmodulin and peptide C20W corresponding to the first 20 amino acids of the calmodulin-binding domain showed that only the C-terminal lobe of calmodulin had high affinity for the peptide (K_D in the nanomolar range).

Calmodulin (CaM)[1] has a two-lobe structure with an overall dumbbell shape and a length of 65 Å. Each lobe has the dimensions 25 Å × 20 Å × 20 Å and contains two EF-hand Ca-binding motifs with a typical helix–loop–helix arrangement (Kretsinger & Nockolds, 1973). Crystal structure studies have shown that the two lobes are separated by a long, α-helix spanning residues 65–92, but recent NMR work indicates a nonhelical, highly flexible structure for residues 78–81 in the center of this helix (Ikura et al., 1991b). This flexibility would permit large conformational changes upon binding of Ca^{2+} with minimal variations in the secondary structure of the N- and the C-terminal domains (Persechini and Kretsinger, 1988). Small-angle X-ray scattering experiments (Seaton et al., 1985; Heidorn & Trewhella, 1988, Kataoka et al., 1991a) have also indicated that the central helix becomes flexible when calmodulin is in solution and adopts a bent conformation in the presence of target molecules. A recent molecular modeling study has shown that the bend in the central helix could explain the cooperativity of Ca^{2+} binding observed in NMR studies of calmodulin complexes (Vorherr et al., 1992). Heteronuclear NMR studies (Ikura et al., 1991a) have provided experimental evidence that conformational changes may indeed occur when CaM is bound to targets. These changes in secondary and tertiary structure are not restricted to the central helix.

The plasma membrane Ca^{2+}-ATPase is regulated directly by CaM. CaM binds to the pump in a Ca^{2+}-dependent way and increases its Ca^{2+} affinity (Niggli et al., 1981). This is important for the control of intracellular free Ca^{2+} which is essential to its signaling function. The calmodulin-binding domain of the Ca^{2+}-pump has been localized in the C-terminal portion of the protein (James et al., 1988; Verma et al., 1988) and displays features that are common to other CaM-binding peptides. Several peptide analogues of this sequence have been studied (Vorherr et al., 1990). CaM-binding peptides from different targets have also been repeatedly investigated, but the structural details of their complexes with CaM are unknown. One complicating factor is that, in addition to CaM, the CaM-binding peptides also change structure upon complex formation. A common observation is helix induction in the peptides, which at the same time cause bending (and other changes) in the structure of CaM.

In this work, photoactivatable, radioactively labeled peptides corresponding to the CaM-binding domain of the plasma membrane Ca^{2+}-pump have been used to locate contact regions in the CaM–peptide complex. The results of the study have shown that the N-terminal portion of the CaM-binding domain of the Ca^{2+}-pump interacts with the C-terminal portion of CaM and that the C-terminal portion of the domain interacts with the N-terminal lobe of CaM.

EXPERIMENTAL PROCEDURES

Materials. CaM was isolated from bovine brain as described by Guerini et al. (1984). It was cleaved by trypsin in the presence of Ca^{2+} according to Walsh et al. (1977) as described elsewhere (Guerini et al., 1984). The fragments were purified according to Toda et al. (1981) on DEAE-cellulose (Whatman, Maidstone, U.K.) using a discontinuous gradient from 50 mM NH_4HCO_3 (buffer A) to 1 M NH_4HCO_3 (buffer B). The purity of the obtained fragments was verified by Tricine–SDS–PAGE according to Schägger and von Jagow (1987), by amino acid analysis, and by partial amino acid sequencing. The C-terminal fragment comprised residues 78–148 whereas the N-terminal fragment contained residues 1–75. Dansylation of the fragments was performed according to Vorherr et al. (1990) showing incorporation of 0.3–0.5 mol of the dansyl moiety/mol of fragment.

Chemicals from Applied Biosystems (Foster City, CA) were used for peptide synthesis, sequencing, and amino acid analysis,

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CaM, calmodulin; DCC, dicyclohexylcarbodiimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Fmoc; 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MLCK, myosin light chain kinase; MOPS, 3-morpholinopropanesulfonic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; tBu, *tert*-butyl; TFA, trifluoroacetic acid.

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Table I: Sequences of the Synthetic Peptides[a]

C20W*	[^{3}H]Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K
C28W*	[^{3}H]Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C28WC*	[^{3}H]Ac-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F*-S-S-S

[a] F* stands for the photoactivatable L-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanyl residue.

except for the amino acid derivatives for peptide synthesis which were obtained from Novabiochem (Laufelfinger, Switzerland) or Bachem AG (Bubendorf, Switzerland). All other reagents and solvents of the highest purity grade available were from Fluka Chemie AG (Buchs, Switzerland).

Synthesis of Peptides C20W, C28W*, and C28WC**. The sequences of the synthetic peptides used in this study are presented in Table I. Details of the synthesis, deprotection, and purification of peptide C28W* ([^{3}H]Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S) are described in Falchetto et al. (1991). The asterisk (*) stands for the (azitrifluoroethyl)phenylalanine in position 9.

Peptide [^{3}H]Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K (C20W*, the asterisk (*) stands for the (azitrifluoroethyl)phenylalanine in position 9) was synthesized on an Applied Biosystems peptide synthesizer Model 431 using the Fmoc/tBu strategy with 1-methyl-2-pyrrolidone for coupling and washing according to the DCC/HOBt standard protocol for the synthesizer. The first amino acid was attached according to the cycle for loading the first amino acid onto the *p*-(hydroxymethyl)phenoxy-polystyrene resin (1.0 mmol/g, 1% divinylbenzene). The peptide was synthesized using Pmc protection for the Arg residues. The fully protected peptide resin (sequence R-G-L-N-R-I-Q-T-Q-I-K) was automatically synthesized using a 10-fold excess of DCC/HOBt-activated Fmoc-amino acid derivatives and a capping cycle employing acetic anhydride after each coupling according to the instructions of the manifacturer. After deblocking of the N-terminus, the diazirine-labeled Fmoc-Phe was coupled manually. The 2-fold excess of diazirine derivative was activated with DCC/HOBt and coupled to 30 μmol of peptide resin in 600 μL of 1-methyl-2-pyrrolidone for 3.5 h. The ninhydrin test according to Kaiser (Kaiser et al., 1970) showed nearly complete reaction (light blue resin beads). Further synthesis prior to capping after coupling the diazirine-labeled Fmoc-Phe was completed again with a 10-fold excess of amino acid derivative. After the last Fmoc deprotection, 75 μL of [^{3}H]acetic anhydride (25 mCi/mL, 50 μmol/mL) in 850 μL methylene chloride was used to radioactively label the N-terminal amino acid. Complete acetylation was achieved with the standard capping protocol. Cleavage was performed in a solution of 1.5 mL of TFA, 400 μL of ethanedithiol, and 80 μL of water for 3 h at room temperature following filtration, precipitation, and washing with diethyl ether. A total of 14.7 mg of purified product was obtained after preparative HPLC using buffers A and B (see below).

The peptide [^{3}H]Ac-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F*-S-S-S (C28WC*, the asterisk (*) stands for the (azitrifluoroethyl)phenylalanine in position 25) was synthesized and purified according to the following procedure: the first amino acid was attached to *p*-(hydroxymethyl)phenoxy-polystyrene (1 mmol/g of resin, 1% divinylbenzene) using dimethylaminopyridine as a catalyst according to the standard procedure for the Applied Biosystems peptide synthesizer Model 431. The Pmc-protected arginine and trityl-protected Asn and Gln were used for this synthesis. The fully protected peptide resin (sequence S-S-S) was automatically synthesized using a 10-fold excess of DCC/HOBt-activated Fmoc amino acid derivatives and a capping cycle employing acetic anhydride after each coupling according to the instructions of the manifacturer. After deblocking of the N-terminus, the diazirine-labeled Fmoc-Phe was coupled manually. A 2-fold excess of diazirine derivative was activated with DCC/HOBt and coupled to 30 μmol of peptide resin in 600 μL of 1-methyl-2-pyrrolidone for 3.5 h. The ninhydrin test according to Kaiser (Kaiser et al., 1970) showed nearly complete reaction (light blue resin beads). Further synthesis prior to capping after coupling the diazirine-labeled Fmoc-Phe was completed again with a 10-fold excess of amino acid derivative. After the last Fmoc deprotection, 75 μL of [^{3}H]-acetic anhydride (25 mCi/mL, 50 μmol/mL) in 850 μL meththylene chloride was used to radioactively label the N-terminal amino acid. Complete acetylation was achieved with the standard capping protocol. A 60-mg sample of acetylated protected peptide resin was cleaved in a solution of 1 mL of TFA, 30 μL of ethanedithiol, 30 μL of thioanisole, and 30 μL of water for 2.5 h at room temperature following filtration, precipitation, and washing with diethyl ether. A total of 16 mg of crude product was obtained. Analytical HPLC indicated that about 65% of the radioactively labeled target peptide was present in the crude material. Amino acid analysis showed the expected ratios among residues (tryptophan was not determined).

HPLC Analysis and Sequencing of the Purified Peptides. Semipreparative HPLC was carried out using Nucleosil reversed-phase material (250 × 55 mm C_8 column 10 μm, 300 Å; Macherey & Nagel, Oensinger, Switzerland). The reversed-phase buffers were (A) 0.1% TFA in water and (B) 0.05% TFA, 50% 1-propanol in water. Purification was performed using a linear gradient from A to B. Analytical control was performed in the following buffer system: (C) 0.1% TFA in water; (D) 0.1% TFA, 70% acetonitrile in water using a 2.1 × 30 mm C_8 column (Aquapore 7 μm; 300 Å) obtained from Applied Biosystems.

The photo-cross-linked products and the digests were separated on a Nucleosil C_{18} column (60 × 4 mm, 3 μm, 120 Å; Macherey & Nagel) or an Aquapore C_4 column (150 × 2.1 mm, 7 μm, 300 Å; Brownlee Laboratories Inc., Santa Clara, CA). One-minute fractions were collected. Radioactivity was measured in Ready Safe Liquid Scintillation Cocktail (Beckman Instruments Inc., Fullerton, CA) with a Beckman LS 1801 counter. HPLC chromatography was carried out using Applied Biosystems and LKB equipment (LKB, Uppsala, Sweden). UV detection was performed at 210 nm. An Applied Biosystems Derivatizer 420A and on-line phenylthiocarbamyl detection with the Model 130A Applied Biosystems analyzer were used for derivatization, separation, and identification of the amino acids. Amino acid analysis was carried out for all synthetic peptides. Sequencing was carried out using an Applied Biosystems 470A sequencer with 120A on-line phenylthiohydantoin detection.

Photocoupling of the Peptides to Calmodulin. All cross-linking experiments using the 28-residue peptides were performed in the following solutions, either in the presence or absence of Ca^{2+}: 20 mM Hepes, 0.1% $C_{12}E_8$ detergent, 1 mM EGTA, pH 6.7–6.8 adjusted after dissolving CaM and the peptide; 20 mM Hepes, 0.1% $C_{12}E_8$, 500 μM $CaCl_2$, pH 6.7–6.8. A CaM concentration of 2 mg/mL was used for

each experiment, whereas in the case of the 28-amino acid peptides the concentration was 0.4 mg/mL. All cross-linking experiments were performed in Eppendorf tubes. Before being mixed with the reaction buffer, peptide C28WC* or C28W* was dissolved in about 30 μL of 1-propanol. The complete reaction mixture was sonicated in the dark for about 5 s. The samples were incubated before irradiation at 37 °C for at least 10 min and mixed several times. Since a precipitate formed after photolysis, a sample from the well-resuspended reaction mixture was dried under nitrogen, dissolved in 70% formic acid, and loaded on the HPLC.

Isolation of the Photo-Cross-Linked Product. Partial separation of the cross-linked products of peptides C20W* and C28W* was achieved by semipreparative HPLC on a C_{18} column (300 Å, 7 μM, 250 × 10 mm; Macherey & Nagel). A linear gradient on buffers C and D was run at a flow rate of 3 mL/min, and detection was performed at 210 nm. The radioactive fractions corresponding to the cross-linked product were used for the digestion experiments.

In the case of peptide C28WC* separation of the photoproduct from free CaM was achieved by preparative 16% Tricine gels (14 × 11.5 cm, 5 mm thick) according to the method of Schaegger and von Jagow (1987). It was verified that such gels can accommodate up to 1.5 mg of total protein. The gels were run at 4 °C for 36 h with a constant voltage of 110 V. Fixing, staining, and destaining were performed in 50% methanol/10% acetic acid for 1 h, 0.025% Serva blue G in 10% acetic acid for 3.5 h, and 10% acetic acid overnight. The bands corresponding to CaM and the CaM–C28WC* complex were cut out of the gel and electroeluted for 6 h at 8 mA/tube using a Bio-Rad electroelutor in the following buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% sodium dodecyl sulfate, 1 mM 1,4-dithio-D,L-threitol. Since the presence of sodium dodecyl sulfate was detrimental to HPLC, it was removed by a two-step dialysis in the following buffers: (1) 50 mM NaH_2PO_4, 4 M urea, pH 7.8, for about 60 h, and (2) 50 mM NaH_2PO_4, 1 M guanidine hydrochloride, pH 7.8, for 48 h. The samples were analyzed as indicated above by mini-Tricine gels and by reversed-phase HPLC. The amount of radioactivity was measured to estimate the cross-linking efficiency and the loss of cross-linked product due to the isolation procedures.

Staphylococcus aureus Strain V8 Protease Digestion of the Cross-Linked Products. In a typical digestion experiment, about 200 μg of protein was concentrated to a final volume of about 300 μL; 12 μL of acetonitrile was added to achieve the following composition of the digestion buffer: 50 mM NaH_2PO_4, 1 M guanidine hydrochloride, 5% acetonitrile, pH 7.8. *S. aureus* V8 protease (purchased from Boehringer Mannheim GmbH, Mannheim, Germany) was added in 3 steps at digestion times 0, 14, and 18 h up to a final ratio V8 protease/protein of 1/10 (w/w). During the digestion (24 h), the sample was incubated at 37 °C.

Cyanogen Bromide Digestion of the Isolated Cross-Linked Calmodulin–C28WC Complex.* Methionine-specific protein digestion was accomplished in 200–300 μL of 70% formic acid with protein quantities of up to 200 μg. Cyanogen bromide was added in a molar ratio of about 300/1 to methionine residues. The sample was then kept under nitrogen in the dark at room temperature for about 24 h. Excess cyanogen bromide was removed by diluting the sample with doubly distilled water to 1 mL and by drying under nitrogen. This step was repeated three times.

Endoproteinase Asp-N Digestion of the Isolated Cross-Linked Calmodulin–C28WC Complex.* The amount of protein and buffer, the concentration and digestion times, and the procedure were exactly the same as for the V8 protease digestion. Asp-N (Boehringer Mannheim GmbH) was added in 3 steps to a final ratio of 1/100 (w/w).

Hydroxylamine Cleavage of the Isolated Cross-Linked Calmodulin–C28WC Complex.* For the precise indentification of the cross-linked amino acid, the radioactive fragments of the Asp-N digestion which contain an Asn-Gly bond were treated with hydroxylamine. The radioactive fraction from the HPLC was lyophilized and then redissolved in 6 M guanidine hydrochloride, 2 M hydroxylamine. The pH was adjusted to 9.0 with a 4.5 M solution of LiOH, and the sample was incubated at 56 °C for 3–4 h. LiOH was added during the reaction to keep the pH at 9.0.

Dansylation of the Calmodulin Fragments. The purified fragments were dansylated in 20 mM NH_4HCO_3, pH 7.5, in the presence of Ca^{2+} as described elsewhere (Vorherr et al., 1990). A total of 0.3–0.5 mol of dansyl chloride/mol of fragment was incorporated.

Fluorescence Measurements. Fluorescence measurements were performed according to Vorherr et al. (1990) with a SPEX Fluorolog 1680 double-wavelength spectrometer connected to a DM1B coordinator (Metuchen, NJ). Quartz cuvettes with a path length of 10 mm and a volume of about 3.5 mL were used. The measurements were performed at room temperature using the following buffer: 20 mM Hepes, pH 7.2, 130 mM KCl, containing either 1 mM Ca^{2+} or 1 mM EGTA. The concentration of the dansylated fragments in all experiments was 240 nM, and the peptide C20W dissolved in doubly distilled water was added to the medium containing the dansylated fragment. The dansyl moiety of the CaM fragments was excited at 340 nm, and the fluorescence enhancement or quenching during the titration was recorded by following the fluorescence emission at 490 and 500 nm for the Ca^{2+}-bound and EGTA-treated states, respectively. Analysis of the data and determination of the affinity constants were performed as previously described (Vorherr et al., 1990).

RESULTS

The calmodulin-binding peptides C20W*, C28W*, and C28WC* (see Table I) carried a photoactivatable group and a radioactive marker. Peptides C20W* and C28W* carried the photolabel in the same position (Phe*9) while the photoactivatable group of peptide C28WC* was located in the C-terminal portion (Phe*25). Previous work has shown (Falchetto et al., 1991) that the derivatization procedure did not significantly decrease the affinity of the peptides for CaM.

Analysis of the Cross-Linked Products of Peptides C20W and C28W*.* The cross-linking of the peptides to CaM and the analysis of the cross-linked products were performed as described in the Materials and Methods section. A band corresponding to the cross-linked peptide–CaM complex appeared in the gels indicating covalent attachment of the peptides (Figure 1). A significant amount of cross-linking was also observed in the presence of EGTA; this was expected since the concentrations used were in the micromolar range and in this concentration range the interaction is not strictly Ca^{2+} dependent (Vorherr et al., 1990). The HPLC analysis of the cross-linked product revealed a pattern similar to that of the complex between CaM and the CaM-binding peptide of myosin light chain kinase (O'Neil et al., 1989). After purification of the cross-linked complexes of peptides C20W* and C28W* by reversed-phase HPLC to eliminate most of CaM and free peptides (data not shown), the cross-linked products were digested with the *S. aureus* strain V8 protease.

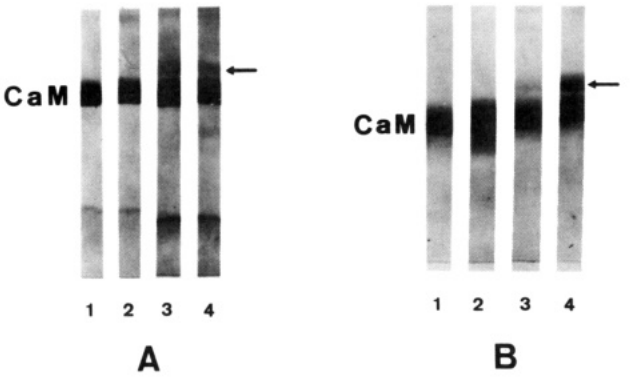

FIGURE 1: Cross-linking experiments using peptides C28W* or C28WC* and calmodulin. The figure shows Serva-blue-stained Tricine–polyacrylamide gels before and after cross-linking (see the Materials and Methods section for details). (A) CaM–C28W*; (B) CaM-C28WC*. Lanes 1 and 3 were in EGTA buffer; lanes 2 and 4 were in the presence of 500 μM $CaCl_2$; lanes 1 and 2 were before photolysis, and lanes 3 and 4 were after photolysis. The arrow indicates the position of the cross-linked products.

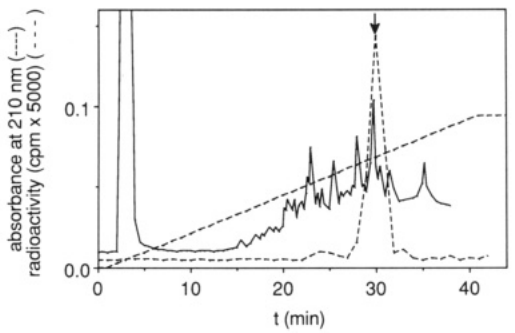

FIGURE 2: Reversed-phase HPLC separation of the fragments obtained after *S. aureus* strain V8 Glu-C protease digestion of the photo-cross-linked product CaM–C20W*. The chromatography was performed on a C_4 column (300 Å, 7 μm, 150 × 2.1 mm) with a linear gradient from 0% to 100% in 40 min. The flow was 300 μL/min, and 30-s fractions were collected. Aliquots of 5 μL were used for scintillation counting (dotted line). The fraction containing most of the radioactivity, indicated by an arrow, was submitted to sequence analysis.

In the case of peptide C20W*, the digest was resolved by reversed-phase chromatography on an Aquapore C4 column (see the Materials and Methods section) using a linear gradient from 0% to 100% buffer B in 40 min. A single radioactive peak (35% of the original input) was detected eluting at around 30 min (Figure 2). In analogy to observations in a previous study (Falchetto et al., 1991), the photolysis products of the free peptide appeared at about the same position as the cross-linked fragments (not shown). The results of the sequence analysis of the labeled fragments are presented in Table II. The two identified sequences belonged to the C-terminal portion of CaM (88–94 and 107–112), but the size of the first fragment could not be identified unambiguously since its repetitive yield was rather low (Table II). Interestingly, fragment 107–112 did not contain an N-terminal Glu residue. In the case of peptide C28W*, the digest was resolved by reversed-phase chromatography on a C_{18} column (see the Materials and Methods section) using a linear gradient from 0% to 100% buffer B in 40 min. Two major radioactive peaks and two minor peaks (3% of the original input) were detected (Figure 3). The late-eluting minor peak corresponded to the undigested cross-linked product. No sequence data could be obtained for these fractions. The early-eluting major radioactive peak corresponded to the sequence of CaM fragment 88–114 (see Table II). Two sequences were identified in the case of the early-eluting minor radioactive peak (88–104 and 105–114). The late-eluting major radioactivity peak corresponded to the cross-linked fragment 88–120. Thus, all sequences cross-linked by peptides C20W* and C28W* belonged to the C-terminal domain of CaM. The identification of Glu residues in the middle of the late-eluting fragment of peptide C28W* and the presence of a small portion of undigested cross-linked product indicated that the digestion had not proceeded to completion.

Table II: Sequencing Yields of the Radioactive Fragments of the V8 Digests after Cross-Linking Peptides C20W* and C28W* to Calmodulin

residue	C20W* V8 (pmol)	C20W* V8 (pmol)	C28W* V8 1 (pmol)	C28W* V8 2 (pmol)	C28W* V8 2 (pmol)	C28W* V8 3 (pmol)
^{88}A	151.1		43.6	x		792.0
^{89}F	39.4		43.1	23.0		538.0
^{90}R	x		13.1	5.6		174.0
^{91}V	28.1		35.7	34.0		678.0
^{92}F	12.0		19.9	21.0		440.0
^{93}D	x		23.5	34.0		380.0
^{94}K	2.0		15.6	15.5		540.0
^{95}D			17.0	23.0		360.0
^{96}G			15.8	22.0		340.0
^{97}N			12.7	12.6		280.0
^{98}G			12.5	15.9		227.0
^{99}Y			8.9	9.1		250.0
^{100}I			8.3	6.4		194.0
^{101}S			12.2	14.0		303.0
^{102}A			7.0	11.2		281.0
^{103}A			9.2	11.8		281.0
^{104}E			6.8	13.1		212.0
^{105}L			6.3		x	183.0
^{106}R			2.7		x	123.0
^{107}H		95.5	1.5		x	35.0
^{108}V		138.1	3.1		34.0	*a*
^{109}M		74.5	1.9		23.0	*a*
^{110}T		42.2	4.5		26.0	*a*
^{111}N		18.9	2.2		x	*a*
^{112}L		18.5	2.6		17.0	49.0
^{113}G		17.4	3.6		22.0	21.0
^{114}E		10.9	2.6		16.0	33.0
^{115}K						x
^{116}L						29.0
^{117}T						44.0
^{118}D						26.0
^{119}E						43.0
^{120}E						45.0
^{121}V						

[a] The amino acid detection in this cycle was disturbed by injection problems.

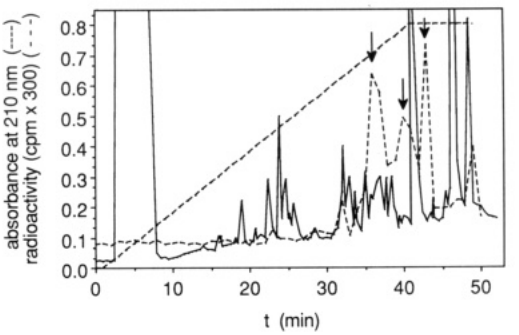

FIGURE 3: Reversed-phase HPLC separation of the fragments obtained after *S. aureus* strain V8 Glu-C protease digestion of the photo-cross-linked product CaM–C28W*. The peptide mixture was separated on a C_{18} reversed-phase column (Nucleosil, 3 μm, 100 Å, 80 × 4 mm). The flow rate was 300 μL/min; detection was at 210 nm (solid line). A linear gradient from 0% to 100% in 40 min was run; 30-s fractions were collected, and 5-μL aliquots were used for scintillation counting (dotted line). The fractions containing most of the radioactivity, indicated by arrows, were submitted to sequence analysis.

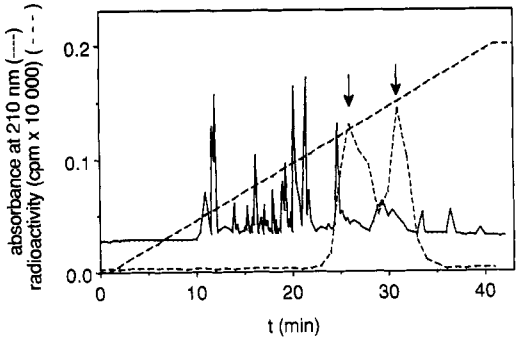

FIGURE 4: Reversed-phase HPLC separation of the fragments obtained after *S. aureus* strain V8 Glu-C protease digestion of the photo-cross-linked product CaM–C28WC*. The chromatography was performed on a C_{18} column (120 Å, 7 μm, 125 × 2.1 mm) with a one-step linear gradient from 0% to 100% in 40 min. The flow was 500 μL/min, and 30-s fractions were collected; 5-μL aliquots were used for scintillation counting (dotted line). The fractions containing most of the radioactivity, indicated by arrows, were submitted to sequence analysis.

*Analysis of the Cross-Linked Product of Peptide C28WC**. Peptide C28WC*, carrying the photoactivatable group in position 25, was incubated with CaM. Analytical gels of the reaction mixtures before and after photolysis (Figure 1B) demonstrated that also in this case a cross-linked product was formed. Peptide C28WC* was more efficient than its N-terminally labeled analogue (Figure 1), and in this case the formation of the cross-linked product was increased by the presence of Ca^{2+}. A yield of up to 23% (determined approximately by excising the gel bands corresponding to the photoproduct and by solubilizing and counting their radioactivity content) was obtained; this permitted purification of the cross-linked product on preparative gels (see the Materials and Methods section). The purified complex was analyzed by reversed-phase HPLC and gel electrophoresis (data not shown); it still contained a small amount of free CaM which apparently did not interfere with the subsequent steps.

The sample containing the purified cross-linked product was divided into three aliquots for digestion with the *S. aureus* V8 Glu-C endoproteinase, the *Pseudomonas fragi* Asp-N endoproteinase, or CNBr, as described in the Materials and Methods section. The HPLC profile of the V8 protease digestion is shown in Figure 4. The radioactivity was recovered in two peaks (see Table III), the sequence of the early-eluting peak being also contained in that of the late peak. A Glu residue in the center of the late-eluting peak suggested that the digestion had not proceeded to completion. Also, the two sequences obtained from the CNBr digestion derived from a broad radioactive peak (Table III): the presence of a Met residue indicated incomplete digestion, probably due to reduced accessibility of the complexed CaM to the reagent. The Asp-N protease digestion resulted in a large fragment, too, which still contained Asp residues. The presence of an Asn–Gly bond in position 61–62 that can cyclize under sequencing conditions practically stopped the sequencing, lowering the yields to values near background (Table III). Nevertheless, in the case of peptide C28WC* the sequencing data showed that a stretch of 9 amino acids spanning from Ala73 to Ser82 most probably contained the site which interacted with Phe in position 25 of the CaM-binding peptide. The region is located within the so-called hydrophobic patch (Persechini et al., 1988) of the N-terminal domain of calmodulin and corresponds to the second Ca^{2+}-binding loop, to helix IV, and to a portion of the postulated central "linker" sequence.

Fluorescence Measurements of the Interaction of the N- and C-Terminal Domains of Calmodulin with Peptide C20W.

Table III: Sequenching Yields of the Radioactive Fragments of the V8 Digests after Cross-Linking Peptides C20W* and C28W* to Calmodulin

residue	CNBr (pmol)	CNBr (pmol)	V8 1 (pmol)	V8 2 (pmol)	Asp-N (pmol)
^{37}R	x				
^{38}S	7.40				
^{39}L	11.60				
^{40}G	12.59				
^{41}Q	7.61				
^{42}N	5.62				
^{43}P	6.06				
^{44}T	9.61				
^{45}E	8.12				
^{46}A	5.82				
^{47}E	10.12				
^{48}L	7.60				
^{49}Q	4.77				
^{50}D	5.06				133.20
^{51}M	3.02				77.70
^{52}I	5.64				75.54
^{53}N	3.01				55.70
^{54}E	4.79				61.00
^{55}V	4.13		17.33		59.50
^{56}D	4.14		17.73		56.70
^{57}A			11.97		51.30
^{58}D			9.67		47.04
^{59}G			17.08		*a*
^{60}N			2.88		7.57
^{61}G			13.51		17.68
^{62}T			3.29		21.58
^{63}I			4.22		17.27
^{64}D			4.67		17.18
^{65}F			3.53		13.79
^{66}P			2.34		8.49
^{67}E			3.87		18.94
^{68}F			2.76	16.62	12.25
^{69}L				16.83	16.15
^{70}T				13.12	15.87
^{71}M				7.60	13.92
^{72}M				10.05	14.06
^{73}A		36.39		9.89	14.32
^{74}R		0.17		5.15	6.58
^{75}K		2.91		3.28	6.44
^{76}M		5.93		6.66	
^{77}K		3.52		3.76	
^{78}D		6.18		8.04	
^{79}T		7.28		5.80	
^{80}D		5.27		6.13	
^{81}S		28.23			

[a] The amino acid detection in this cycle was disturbed by injection problems.

Peptide C20W, corresponding to the N-terminal portion of the CaM-binding domain of the Ca^{2+}-pump, was studied in fluorescence experiments with the dansylated, isolated N- and C-terminal domains of calmodulin. The peptide had been previously shown to bind to intact CaM with an affinity (K_D of about 11 nM; Vorherr et al., 1990a) very close to that of the native ATPase (K_D of 4 nM). The studies demonstrated that the peptide was not able to bind to the N-terminal half of CaM with a K_D in the nanomolar range. As can be seen from Figure 5, complex formation, which in this case resulted in fluorescence enhancement, failed to reach saturation, even at a peptide to N-terminal CaM fragment ratio of 100/1 in the presence of Ca^{2+}. By contrast, Figure 6 shows that the binding of the C-terminal half of CaM to the peptide, resulting in this case in the quenching of the fluorescence, had a dissociation constant of about 400 nM (see the insert of the figure). As can be seen from the figure, at a 1/1 ratio of peptide C20W to the dansylated C-terminal fragment of CaM, the formation of the complex was more than 70% completed in the presence of Ca^{2+}. No complex formation was observed in the presence of EGTA.

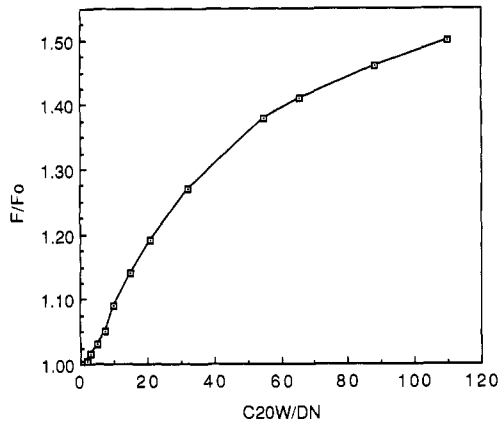

FIGURE 5: Interaction of the N-terminal half of CaM with peptide C20W. The dansylated N-terminal half of CaM (240 nM) was titrated with the CaM-binding peptide C20W in the presence of Ca^{2+}. Excitation was performed at 340 nm, and fluorescence emission was recorded at 490 nm. The relative fluorescence intensities are plotted against the ratio of the total concentration of peptide C20W and the total concentration of the dansylated fragment of CaM. The data points correspond to fluorescence intensities integrated over a total time of 5 s. DN represents the dansylated N-terminal half of CaM.

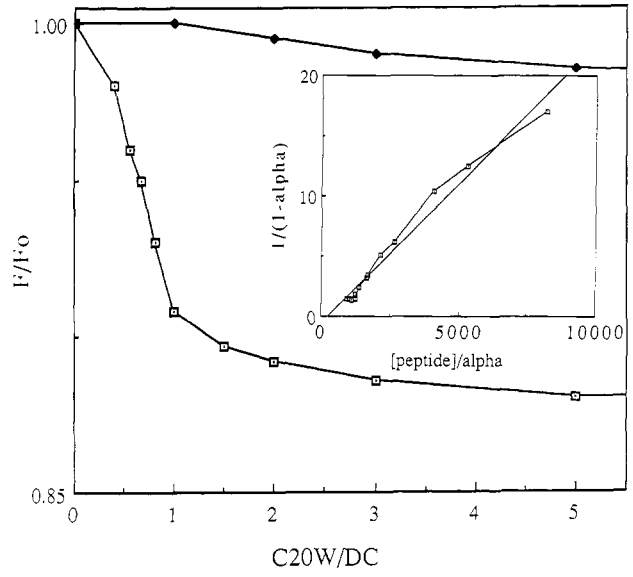

FIGURE 6: Titration of the dansylated C-terminal half of CaM (240 nM) with the calmodulin binding peptide C20W in the presence of Ca^{2+} (□) or EGTA (◆), respectively. Excitation was performed at 340 nm, and the fluorescence emission was observed at either 490 nm (Ca^{2+}) or 500 nm (EGTA) essentially in the same way as described in the legend to Figure 5. The relative fluorescence intensities are plotted against the ratio of the total concentration of peptide C20W and the total concentration of the dansylated fragment of CaM. DC represents the dansylated C-terminal half of CaM. The insert shows the derivation of the affinity constant of the peptide C20W for the C-terminal half of CaM. The fractional degree of saturation of the dansylated fragment (α) was calculated as described in detail by Vorherr et al. (1990a). The plot $1/(1 - \alpha)$ against the free concentration (nanomolar) of peptide divided by α results in a straight line if a 1:1 complex is formed. The zero intercept on the x-axis refers to the total concentration of the dansylated fragment (240 nM) and serves as a control. The reciprocal of the slope gives the affinity constant. The calculated K value was 430 ± 70 nM.

DISCUSSION

This study has characterized the contact regions between the N- and C-terminal domains of CaM and the CaM-binding domain of the plasma membrane Ca^{2+}-pump. The first portion of the work relates to previous findings on a peptide from the CaM-binding domain of MLCK and on model amphiphilic peptides (O'Neil et al., 1989). O'Neil et al. (1989) replaced a tryptophan side chain in the peptides with a photoactivatable *p*-benzoyl group. Since *p*-benzoyl photolabeling is strongly specific for C–H bonds of tertiary carbons, while the carbenes produced from the diazirines used in the present study react with any neighboring polar or nonpolar group, the differences in the photolabeling pattern can be rationalized. One advantage of carbene-generating groups is their high reactivity, facilitating binding to all residues close in space to the side chain carrying the carbene. However, carbene photolabeling only rarely permits the identification of single amino acid residues involved in the interaction. Even if not permitting the assignment of single residues, the photolabeling method used in this study has led to the identification of interacting spatial domains of very limited extension.

Peptide C28WC* was more efficient as a cross-linker than peptide C28W*. Inactivation of the carbene due to a more pronounced exposure of the photolabeling group in peptide C28W* to the solvent could explain the lower cross-linking efficiency of the latter, although the difference could also have been due to the tighter binding of Phe*25.

The cross-linked domains in the complexes CaM–C28W* and CaM–C28WC* could be discussed with respect to the crystal structure of CaM. Two hydrophobic clefts in the two lobes of CaM have been identified in the crystal structure refined to 2.2 Å (Babu et al., 1988). A high degree of direct homology (71%) was observed between the two clefts, whose overall dimensions were 10 Å and 12.5 Å with a depth of 9.5 Å. The pocket was found to be composed of 14 residues, among them two of the five aromatic amino acids present in each lobe of CaM. In addition, 4 of the 5 aromatic residues in each lobe formed a cluster in which pairs of aromatic rings were oriented perpendicular to each other, i.e., Phe89 and Tyr138 or Phe92 and Phe141, respectively, in the C-terminal half of CaM (Babu et al., 1988). Since hydrophobic patches of CaM are known to become exposed upon binding of Ca^{2+} (La Porte et al., 1980; Krebs et al., 1984), these residues are likely to be involved in target binding.

Peptide C28W* labeled the C-terminal domain of CaM. Closer inspections of the sequences obtained suggests that residues Phe92 and Met109 of the C-terminal hydrophobic cleft become labeled since their yield during sequencing decreased drastically. The suggestion is corroborated by the crystal structure which indicates that Phe92 and Met109 are less than 10 Å apart in space. When peptide C28WC* was used, the N-terminal hydrophobic pocket interacted with Phe25 of the calmodulin-binding peptide. Peptide C28WC* probably labeled Met71, since a drop in the yield was observed for this residue during sequencing of the various labeled fragments. (Arg74 and Lys75 also showed low repetitive yields, but these residues are normally underestimated during sequencing runs.) The crystal structure of CaM shows that these amino acids with low repetitive yields are spatial neighbors, although they are separated in the primary structure by up to 17 residues. The side chains of Val55, Ile63, Phe68, Met71, and Met72 point to each other and form a hydrophobic pocket in the N-terminal lobe of CaM which could easily accommodate an aromatic side chain. On the basis of the three-dimensional structure of CaM, it could therefore be suggested that Phe*25 of the CaM-binding peptide became inserted in the hydrophobic pocket and as a consequence amino acids in the environment became labeled. A similar interpretation could be offered for the labeling of the C-terminal hydrophobic pocket of CaM by C28W*. Interestingly, Met71 had been previously shown to be labeled by *p*-benzoylphen-

ylalanine located at position 13 of the C-terminal portion of CaM-binding peptides, whereas peptides carrying the photolabel in position 3 labeled Met144 of CaM (O'Neil et al., 1989). While this paper was under consideration, the solution structure of the complex between a peptide corresponding to the CaM-binding domain of MLCK (M13) and CaM (Ikura et al., 1992) was published; it agrees with the interpretation suggested here. The study emphasizes the importance of hydrophobic amino acids in the N- and C-terminal part of the peptide as anchors interacting with the hydrophobic pockets of the C- and the N-terminal halves of CaM, respectively. It is of interest that the tryptophan residue in the N-terminal portion of M13 is in close contact with residues F89, F92, F141, and M109 of CaM, whereas I20 in the C-terminal portion interacts, among others, with M71 of CaM.

The results of the fluorescence work with peptide C20W agree with the discussion above: the 20-residue peptide only interacted efficiently (i.e., with a K_D in the nanomolar range) with the C-terminal half of CaM. The results of the photolabeling experiments with peptide C28WC* emphasize the role of the 8 C-terminal residues of the peptide in its interaction with the N-terminal half of CaM. All this is in line with recent findings in which peptide C28W was able to "bridge" the isolated N- and C-terminal domains of CaM suggesting contact regions in both domains (Yazawa et al., 1992). This conclusion is further corroborated by small-angle X-ray scattering data showing that C24W, equivalent to C28W but lacking the first 4 amino acids, contracted CaM to a more globular structure (Kataoka et al., 1991b). Interestingly, C20W was found not to affect the extended, dumbbell-like structure of CaM, since it bound preferentially to its C-terminal half (Kataoka et al., 1991b; Vorherr et al., 1990). It thus seems safe to conclude that the primary recognition site for the CaM-binding domain of the Ca^{2+}-pump is located in the C-terminal half of CaM, although the N-terminal portion of the latter is needed for high-affinity binding and full cooperativity of the system. This conclusion is also supported by former observations that the C-terminal domain of CaM can fully activate the plasma membrane Ca^{2+}-pump, but with 100-fold lower efficiency with respect to intact CaM (Guerini et al., 1984).

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