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# PURIFICATION AND PEPTIDE MAPPING OF CALMODULIN AND ITS CHEMICALLY MODIFIED DERIVATIVES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ALLAN S. MANALAN*, DIANNE L. NEWTON and CLAUDE B. KLEE
*Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)*

## SUMMARY

Methods were developed for the isolation and peptide mapping of calmodulin and its chemically modified derivatives by reversed-phase high-performance liquid chromatography (HPLC). Calmodulin and its guanidinated, iodinated, and performic acid-oxidized derivatives can be isolated on alkylphenyl columns by using gradients of acetonitrile in 10 m*M* potassium phosphate, pH 6.0, 2 m*M* EGTA. Peptide mapping by HPLC, following complete digestion of the proteins with clostripain, allows identification of the modified amino acids residues. Clostripain peptides are eluted in the order 87–90, 75–86, 91–106, 107–126, 127–148, 107–148, 1–37, and 38–74. Performic acid oxidation of methionines decreases the retention times of the modified peptides, whereas iodination of tyrosines or guanidination of lysines increases retention times of modified peptides. These HPLC methods are applicable to the identification of specific modifications of calmodulin, allowing the assessment of the role of individual amino acid residues in determining the unique physical, chemical, and spectroscopic properties of this ubiquitous intracellular calcium-binding protein.

## INTRODUCTION

Calmodulin, a ubiquitous intracellular $Ca^{2+}$-binding protein, is a multifunctional intracellular receptor, which mediates many biochemical effects of $Ca^{2+}$ (see refs. 1–5 for reviews). Upon binding $Ca^{2+}$, calmodulin undergoes conformational changes, which produce specific interaction site(s) recognized by many different proteins. The unique ability of calmodulin to bind to and stimulate the activity of a large number of enzymes allows calmodulin to play an important role in regulating cellular function. Complete understanding of mechanisms by which calmodulin transduces the $Ca^{2+}$ signal requires identification of calmodulin-regulated proteins and elucidation of the molecular mechanisms involved in calmodulin binding and activation of its target enzymes.

The use of radiolabeled calmodulin derivatives in conjunction with crosslinking[6,7], azido labeling[8,9], and gel overlay methods[10–12] has facilitated identification of a variety of calmodulin binding proteins. The development of fluorescent and azido

derivatives of calmodulin has provided the basis for more precise characterization of the interaction of the protein with metal ligands, target proteins, and antagonists[7–9,13–16]. In addition, covalent adducts of calmodulin with calmodulin antagonists have been used to characterize the calmodulin interaction sites for proteins under its control[17,18]. In experiments in which calmodulin derivatives are used to study structural–functional relationships, the complete interpretation of experimental results requires knowledge of the intramolecular distribution of modifying groups in the calmodulin derivative. The present report describes a reversed-phase HPLC peptide mapping procedure, which has proven useful in the characterization of a variety of radiolabeled calmodulin derivatives. In addition, a procedure for small-scale purification of radiolabeled calmodulin and its derivates by reversed-phase high-performance liquid chromatography is presented.

## MATERIALS AND METHODS

Calmodulin was purified from bovine testes by modification of previously published methods[19,20]. Clostripain was purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). CAPP 2-Chloro-10-(3-aminopropyl)phenothiazine–HCl (CAPP) was kindly provided by Dr. Albert Manian, Psychopharmacology Research Branch, National Institute of Mental Health. CAPP–Affigel-10 was prepared as previously described[21].

### *Purification of [$^{35}$S]calmodulin*

[$^{35}$S]Calmodulin was isolated from NIH 3T3 cells that had been incubated for 20 h in methionine-free medium, supplemented with [$^{35}$]methionine (Amersham). Cells were disrupted by sonication in buffer, consisting of 0.33 *M* sucrose, 50 m*M* Tris–HCl (pH 7.5), 1 m*M* magnesium chloride, 10 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), 1 μg/ml pepstatin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 75 μg/ml phenylmethylsulfonyl fluoride (PMSF), and the supernatant was isolated after centrifugation. The supernatant was made 2 m*M* in calcium chloride, and applied to a 3.7 × 0.5 cm column of CAPP–Affigel-10, equilibrated with 0.04 *M* Tris–HCl (pH 7.5), 0.05 *M* sodium chloride, 1 m*M* magnesium chloride, and 0.5 m*M* dithiothreitol (DTT). The column was washed with equilibration buffer, followed by a wash with the same buffer containing 0.5 *M* sodium chloride. [$^{35}$S]Calmodulin was eluted from the column with the 0.5 *M* sodium chloride buffer containing 2 m*M* ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) instead of calcium chloride. The EGTA eluate was applied to a μBondapak alkylphenyl column (30 cm × 3.9 mm, particle size, 10 μm, Phenyl column, Waters Assoc., Milford, MA, U.S.A.), equilibrated with 0.01 *M* potassium phosphate (pH 6.1), 2 m*M* EGTA, and 5% aqueous acetonitrile (Buffer A) and eluted at room temperature with a 20 min linear gradient from 100% buffer A to 35% buffer A and 65% acetonitrile at a constant flow-rate of 1.5 ml/min. [$^{35}$S]Calmodulin was eluted as a sharp peak at 12.2 min (Fig. 1). Fractions containing [$^{35}$S]calmodulin were pooled, flash-evaporated, resuspended in 0.1 ml of 0.1 *M* ammonium bicarbonate, and desalted by chromatography on a 6 × 0.6 cm Sephadex G-25 column, equilibrated and eluted with 0.1 *M* ammonium bicarbonate. Fractions containing protein were pooled, lyophilized, and stored at −70°C.

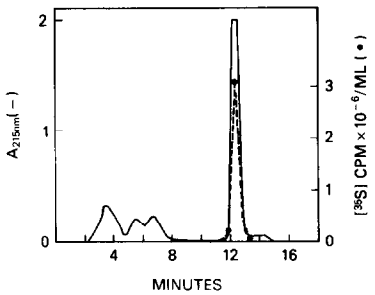


Fig. 1. Isolation of [$^{35}$S]calmodulin by reversed-phase HPLC. The EGTA eluate of a CAPP–Affigel column was applied to a μBondapak alkylphenyl column and eluted as described in the text. Calmodulin and its associated radioactivity (●) were eluted as a single peak at 12.2 min.

*Performic acid oxidation of calmodulin*

Performic acid oxidation was performed by the method of Hirs[22]. Under these reaction conditions, oxidation of methionyl residues, identified as methionine sulfone by amino acid analysis, was essentially quantitative (Table I). No modification of tyrosines was detected by amino acid analysis.

TABLE I

AMINO ACID COMPOSITION OF PEPTIDES GENERATED BY DIGESTION OF PERFORMIC ACID OXIDIZED CALMODULIN WITH CLOSTRIPAIN

The predicted composition of each peptide, calculated from the amino acid sequence of calmodulin[37], is indicated in parentheses. TML = Trimethyllysine.

| | *Peptide* | | | | | |
|---|---|---|---|---|---|---|
| | *87–90* | *75–86* | *91–106* | *107–148* | *38–74* | *1–37* |
| | *Elution time (min)* | | | | | |
| | *10.6* | *11.9* | *22.4* | *30.7* | *35.9* | *36.9* |
| Lys | 0.1 (0) | 1.9 (2) | 1.2 (1) | 1.3 (1) | 0.2 (0) | 3.0 (3) |
| His | 0 (0) | 0 (0) | 0.1 (0) | 1.0 (1) | 0.1 (0) | 0 (0) |
| Arg | 0.9 (1) | 1.0 (1) | 1.0 (1) | 1.1 (1) | 1.0 (1) | 0.9 (1) |
| Asx | 0.2 (0) | 2.2 (2) | 2.5 (3) | 7.2 (7) | 6.6 (7) | 4.3 (4) |
| Thr | 0.1 (0) | 1.4 (1) | 0.5 (0) | 3.1 (3) | 3.2 (3) | 4.6 (5) |
| Ser | 0.1 (0) | 1.1 (1) | 1.0 (1) | 0.4 (0) | 1.1 (1) | 1.1 (1) |
| Glx | 1.0 (1) | 3.0 (3) | 1.0 (1) | 9.0 (9) | 5.4 (6) | 6.6 (7) |
| Pro | 0.1 (0) | 0 (0) | 0.2 (0) | 0.2 (0) | 2.0 (2) | 0.3 (0) |
| Gly | 0.1 (0) | 0.3 (0) | 2.0 (2) | 3.1 (3) | 3.1 (3) | 3.0 (3) |
| Ala | 1.0 (1) | 0.1 (0) | 1.8 (2) | 2.3 (2) | 3.0 (3) | 3.1 (3) |
| Val | 0.1 (0) | 0 (0) | 0.9 (1) | 3.4 (4) | 1.1 (1) | 0.8 (1) |
| Met | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0.1 (0) | 0 (0) |
| Ile | 0 (0) | 1.0 (1) | 1.0 (1) | 1.7 (2) | 1.8 (2) | 2.0 (2) |
| Leu | 0.1 (0) | 0.1 (0) | 1.2 (1) | 2.3 (2) | 3.0 (3) | 3.0 (3) |
| Tyr | 0 (0) | 0.1 (0) | 0.8 (1) | 1.0 (1) | 0.2 (0) | 0.1 (0) |
| Phe | 1.0 (1) | 0.1 (0) | 0.9 (1) | 1.0 (1) | 2.0 (2) | 3.0 (3) |
| TML | 0 (0) | 0 (0) | 0 (0) | 1.0 (1) | 0 (0) | 0 (0) |
| Methioninesulfone | 0 (0) | 1.0 (1) | 0 (0) | 3.2 (4) | 2.6 (3) | 0.9 (1) |

*Guanidination of calmodulin*

(a) Guanidination of calmodulin was performed with O-methylisourea by a procedure originally described for guanidination of ribonuclease[23]. Calmodulin (15.3 mg lyophilized powder) was dissolved in 3.1 ml of 0.5 *M* O-methylisourea–HCl (pH 10.5 with sodium hydroxide) and incubated for 25 min at 2°C. The reaction was terminated by subjecting the incubation mixture to gel filtration on a 17 × 1.5 cm Sephadex G-10 column, equilibrated and eluted with 0.05 *M* ammonium bicarbonate. Fractions containing protein were pooled, lyophilized, and stored at −70°C. The guanidinated protein contained 2.0 lysines and 5.4 homoarginines/mol, identified by amino acid analysis.

(b) [$^{14}$C]Guanidinated calmodulin was prepared by the method of Klee and Richards[23] with O-[$^{14}$C]methylisourea, prepared from barium [$^{14}$C]cyanamide (Rose Chem. Products).

*Iodination of calmodulin*

Iodination of calmodulin with non-radioactive sodium iodide was performed by a previously described glucose oxidase–lactoperoxidase method[24].

*Clostripain digest*

Clostripain was activated at a concentration of 6.6 mg/ml in 0.2 *M* sodium phosphate buffer (pH 7.8), containing 10 m*M* DTT, overnight at 4°C[25]. Calmodulin or modified calmodulin was dissolved in 0.05 *M* sodium phosphate buffer (pH 7.8), containing 2 *M* urea, 1 m*M* calcium chloride, and 0.5 m*M* DTT, at a concentration of 15–90 $\mu M$. An aliquot of clostripain was added to bring the concentration of clostripain to 0.11 mg/ml (2.2 $\mu M$), and the digestion mixture was incubated at 37°C for 3 h. A second aliquot of clostripain was added, and digestion at 37°C was continued for an additional 3 h. Digestion was then stopped by the addition of N$\alpha$-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (final concentration 10 $\mu M$). Digestion mixtures were stored at −70°C.

*HPLC peptide mapping*

The HPLC system consisted of two Model 6000 A solvent delivery systems, equipped with a Model 660 solvent programmer, a Model 450 variable-wavelength detector, a Model 440 fixed-wavelength detector, and a data module for recording and processing data (all from Waters Assoc.). All aqueous solvents were filtered through 0.45-$\mu$m Millipore filters (Milford, MA, U.S.A.) and degassed under vacuum with stirring for 30 min prior to use. Aqueous solvents were further purified by passage through a 61 cm × 7.8 mm $C_{18}$ Porasil guard column (Waters Assoc.) placed between the aqueous solvent pump and the mixing chamber. Calmodulin peptides, derived by digestion with clostripain were separated on a $\mu$Bondapak $C_{18}$ reversed-phase column (30 cm × 3.9 mm; particle size 10 $\mu$m, Waters Assoc.). The fractionation was performed as described for the separation of peptides of intestinal $Ca^{2+}$-binding protein by Fullmer and Wasserman[26]. Digests were loaded onto the column, equilibrated with 0.1% orthophosphoric acid (pH 2.2) (solvent A). The peptides were eluted at room temperature with a 50-min linear gradient from 100% solvent A to a mixture of 50% solvent A and 50% acetonitrile at a constant flow-rate of 2 ml/min. Column eluates were monitored simultaneously for UV absorption at 215 and 280

nm to identify the various components. Eluates were collected in 0.3-min (0.6-ml) fractions. Fractions containing each peptide were pooled, flash-evaporated, and the peptides were identified by their amino acid composition. For analysis of radiolabeled samples, an aliquot (0.4 ml) of each fraction was suspended in 8 ml Aquasol (New England Nuclear, Boston, MA, U.S.A.) for scintillation counting.

*Amino acid analysis*

Analyses were performed on a Beckman Model 120C amino acid analyzer (Beckman Instruments, Irvine, CA, U.S.A.) as previously described[19].

## RESULTS

*Small-scale purification of [$^{35}$S]calmodulin*

[$^{35}$S]Calmodulin was prepared from NIH 3T3 cells, radiolabeled with [$^{35}$S]-methionine. [$^{35}$S]Calmodulin was isolated from cell extracts by CAPP–Affigel chromatography. Reversed-phase HPLC on an alkylphenyl column was used as the final step in the purification. [$^{35}$S]Calmodulin was eluted as a single peak at 12.2 min, well separated from other UV-absorbing constituents (Fig. 1). Recovery of radioactivity was greater than 90%. The HPLC-purified [$^{35}$S]calmodulin migrated as a single Coomassie Blue-staining band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and a corresponding single band of radioactivity was demonstrated by gel autoradiography. This reversed-phase HPLC method has also been employed as a final step in the purification of [$^{14}$C]guanidinated calmodulin and [$^{14}$C]-acetylated calmodulin[27]. Under the elution conditions described, calmodulin and these radiolabeled derivatives are not resolved by the alkylphenyl column, and are eluted as a single peak (data not shown). Calmodulin, isolated by this method, is recovered in a high yield and retains full biological activity[28].

*HPLC peptide mapping of calmodulin*

Because of the reported specificity for arginyl residues[29], clostripain, a sulfhydryl protease from *Clostridium histolyticum*, was selected to prepare digests of cal-

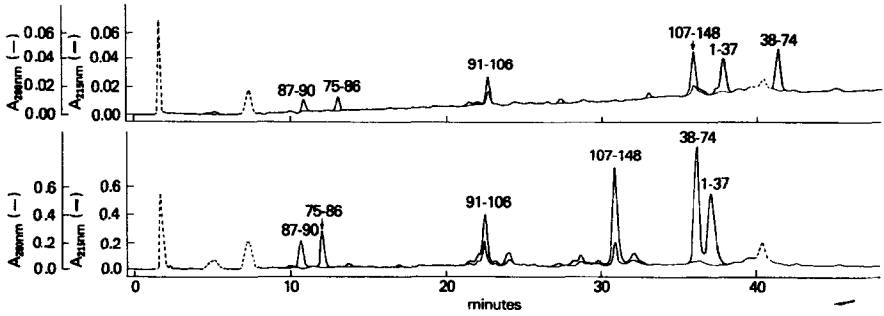


Fig. 2. Comparison of HPLC profiles of peptides, generated by digestion of calmodulin (top panel), and performic acid oxidized calmodulin (bottom panel), with clostripain. Digestion mixtures corresponding to 35 μg calmodulin (top) and 570 μg performic acid-oxidized calmodulin (bottom) were applied to a $C_{18}$ μBondapak column and eluted, as described in the text. Peptides were identified by amino acid analysis (Table I). Broken lines indicate the UV-absorbing peaks contributed by additional reagents present in the digestion mixture.

modulin and its derivatives. The calmodulin peptides were then rapidly and reproducibly separated by reversed-phase HPLC on a μBondapak $C_{18}$ column (Waters Assoc.). By this method, six peptides, derived from calmodulin, were clearly separated (Fig. 2, upper panel). Only the peptides with retention times of 23 and 36 min exhibited significant absorption at 280 nm, indicating that these peptides contained either of the two tyrosyl residues present in calmodulin. The amino acid composition of each peptide was determined, allowing identification of each peptide within the sequence of calmodulin. The entire sequence of calmodulin was found to be contained in the six peptides 1–37, 38–74, 75–86, 87–90, 91–106, and 107–148. This pattern of digestion corresponded with the reported specificity of clostripain for peptide bonds on the carboxyl side of arginyl residues[29]. The peptide bond at Arg-126 appeared relatively resistant to proteolysis. Only limited and nonreproducible cleavage, which yielded peptides 107–126 and 127–148, was achieved (compare Fig. 2, upper panel, with Fig. 3, lower panel). The small UV-absorbing peaks which are eluted immediately following peptides 127–148 and 107–148 appear to represent peptides 127–146 and 107–146, derived from the des(Ala,Lys)calmodulin present in the calmodulin preparation[30].

Treatment of calmodulin with performic acid resulted in oxidation of methionyl residues to methionine sulfone, as documented by amino acid analysis (Table I). Following digestion with clostripain, six major calmodulin peptides were separated by HPLC (Fig. 2, lower panel). Identification of each peptide was again accomplished

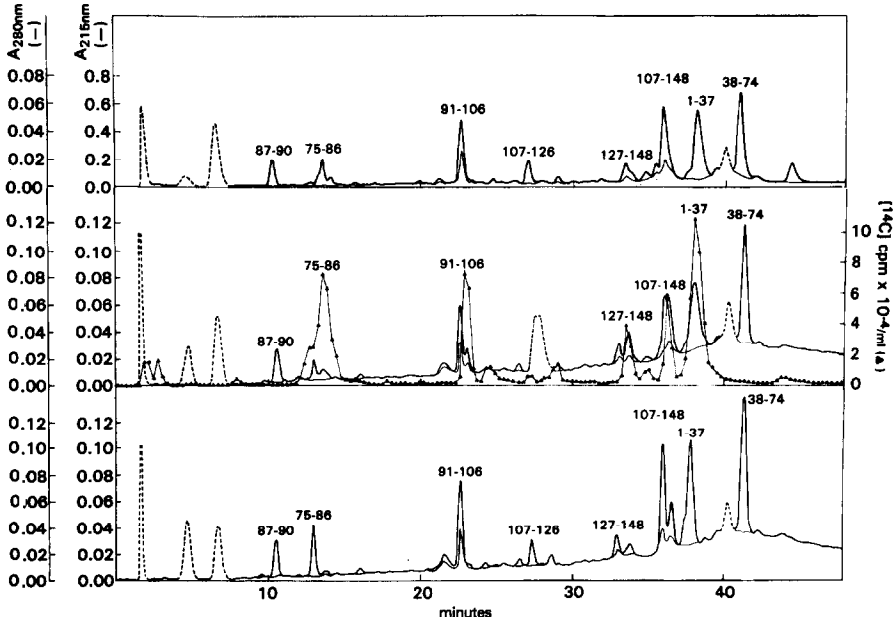


Fig. 3. HPLC elution profiles of clostripain digests of calmodulin (58 μg, bottom panel), [$^{14}$C]guanidinated calmodulin (48 μg, 2.3 lysyl residues modified/mol, middle panel) and guanidinated calmodulin (600 μg, 5.4 lysyl residues modified/mol, top panel). Identification of peptides of guanidinated calmodulin was accomplished by amino acid analysis (Table II). Broken lines indicate UV-absorbing peaks, contributed by additional reagents in digestion mixtures.

TABLE II

AMINO ACID COMPOSITION OF GUANIDINATED PEPTIDES GENERATED BY DIGESTION OF GUANIDINATED CALMODULIN WITH CLOSTRIPAIN

The composition of each peptide predicted from the amino acid sequence of calmodulin[37] is indicated in parentheses. TML = Trimethyllysine.

| | *Peptide* | | | | |
|---|---|---|---|---|---|
| | *75–86* | *91–106* | *127–148* | *107–148* | *1–37* |
| | *Elution time (min)* | | | | |
| | *13.6* | *23.7* | *35.8* | *39.1* | *41.5* |
| Lys | 0.3 (2) | 0.1 (1) | 0 (1) | 0 (1) | 0 (3) |
| His | 0.1 (0) | 0 (0) | 0.1 (0) | 1.0 (1) | 0 (0) |
| Arg | 1.0 (1) | 1.0 (1) | 0.4 (0) | 0.7 (1) | 0.9 (1) |
| Asx | 2.2 (2) | 3.1 (3) | 4.0 (4) | 7.7 (7) | 4.8 (4) |
| Thr | 1.0 (1) | 0 (0) | 1.2 (1) | 2.8 (3) | 4.9 (5) |
| Ser | 1.1 (1) | 1.0 (1) | 0.8 (0) | 0.3 (0) | 1.1 (1) |
| Glx | 3.1 (3) | 1.0 (1) | 4.7 (5) | 9.0 (9) | 6.7 (7) |
| Pro | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Gly | 0.3 (0) | 2.0 (2) | 2.8 (2) | 3.4 (3) | 3.2 (3) |
| Ala | 0.1 (0) | 2.0 (2) | 1.9 (2) | 2.0 (2) | 2.9 (3) |
| Val | 0.1 (0) | 0.9 (1) | 1.8 (2) | 3.6 (4) | 1.0 (1) |
| Met | 1.0 (1) | 0 (0) | 1.4 (2) | 4.1 (4) | 0.9 (1) |
| Ile | 1.0 (1) | 0.9 (1) | 1.1 (1) | 1.8 (2) | 1.9 (2) |
| Leu | 0.1 (0) | 1.0 (1) | 0.7 (0) | 2.2 (2) | 3.2 (3) |
| Tyr | 0 (0) | 0.9 (1) | 1.0 (1) | 1.0 (1) | 0.1 (0) |
| Phe | 0 (0) | 0.9 (1) | 1.1 (1) | 1.0 (1) | 2.9 (3) |
| TML | 0 (0) | 0 (0) | 0 (0) | 0.9 (1) | 0 (0) |
| Homoarg | 1.7 | 1.0 | 0.7 | 0.9 | 2.6 |

by amino acid analysis (Table I). Amino acid analysis of each peptide confirmed the quantitative conversion of all methionyl residues to methionine sulfone. Comparisons of the peptide elution profiles for calmodulin and performic acid oxidized calmodulin revealed that retention times for all methionine-containing peptides (75–86, 107–148, 1–37 and 38–74) were decreased as a result of oxidation of methionyl residues. Peaks with retention times corresponding to unmodified methionine-containing peptides completely disappeared, indicating that oxidation of methionyl residues was complete. Retention of peptides 38–74 and 107–148, which contain 3 and 4 methionyl residues, respectively, were most substantially reduced (5 min). However, the retention times of peptides 1–37 and 75–86, each of which contains only one methionyl residue, were clearly decreased (1 min) by performic acid oxidation. This emphasizes the high sensitivity of retention time to even a single covalent modification. The elution times for peptides 87–90 and 91–106, which do not contain methionyl residues, were unaltered by performic acid oxidation.

The peptides generated by digestion of guanidinated calmodulin (5.4 homoarginines, 2.0 lysines/mol) with clostripain were separated by HPLC (Fig. 3, top panel). Peptides were identified by amino acid composition (Table II), as well as by their characteristic elution times. The pattern of digestion of guanidated calmodulin was

the same as for unmodified calmodulin, demonstrating that the efficiency and specificity of clostripain digestion were not altered by guanidination of lysyl residues. Elution of the resultant peptides was sensitive to covalent modification. Guanidination resulted in delayed elution of all lysine-containing peptides: 75–86, 91–106, 127–148 and 1–37. Retention times of peptides 87–90, 107–126 and 38–74, which contain no lysyl residues, were not altered.

The relative susceptibility of the seven lysyl residues of calmodulin to guanidination was monitored by examination of the distribution of the $^{14}$C-radiolabel among the calmodulin peptides following limited guanidination of calmodulin (2.3 lysyl residues modified/mol) (Fig. 3, middle panel). As a result of this limited guanidination, lysine-containing peptides tended to be eluted in multiple peaks. When these peaks were clearly resolved (91–106, 127–148 and 107–148), the earliest eluted fraction, which contained no radioactivity, was eluted at a position identical to that of the corresponding peptide from unmodified calmodulin. The radioactive peaks were eluted late at positions corresponding to peptides of completely guanidinated calmodulin. Peptide 75–86, which contains two lysyl residues (Lys-75 and Lys-77), exhibited a more complex pattern of elution, consistent with the presence of completely and partially modified, as well as unmodified peptides. Recovery of radioactivity in the [$^{14}$C]guanidinated peptides was in the range of 85–95% of the radioactivity applied. Similar recovery of radioactivity was observed with clostripain digests of [$^{35}$S]calmodulin (80–95%) (data not shown). Preliminary results indicate that recovery of proteins under these conditions is also in the range of 80–95% (see ref. 30).

*Applications of HPLC peptide mapping of modified calmodulin*

The HPLC peptide mapping technique has been used to characterize the intramolecular distribution of radioactivity in preparations of radiolabeled calmodulin. Analysis of a clostripain digest of [$^{3}$H]calmodulin (New England Nuclear) is presented in Fig. 4. The HPLC peptide map demonstrates a major peak of radioactivity, eluted at 26.2 min, indicating that radiolabel is confined primarily to a single site within calmodulin. The large absorbance at 280 nm of the radioactive peak suggests that Bolton–Hunter reagent was employed in the radiolabeling process. The smaller

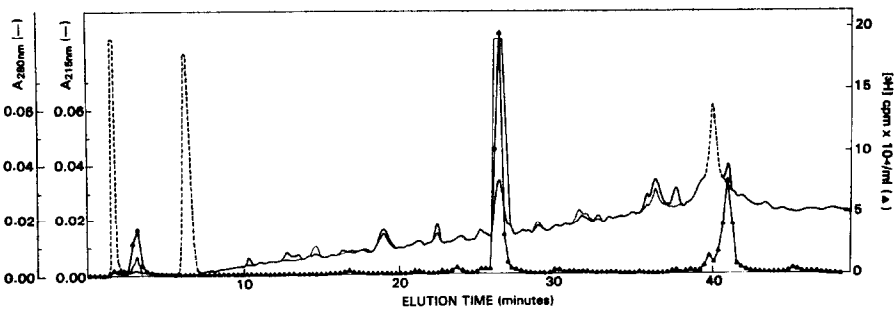


Fig. 4. HPLC peptide map of [$^{3}$H]calmodulin, digested with clostripain. A clostripain digest of [$^{3}$H]calmodulin (15 $\mu$g) was applied to a $\mu$Bondapak $C_{18}$ column and eluted as described in the text. The unmodified calmodulin peptides can be identified by their characteristic retention times (compare Fig. 2, upper panel, with Fig. 3, lower panel). A major peak of radioactivity and absorbance at 280 nm is observed at 26.2 min, indicating that radiolabel is confined primarily to a single site within calmodulin.

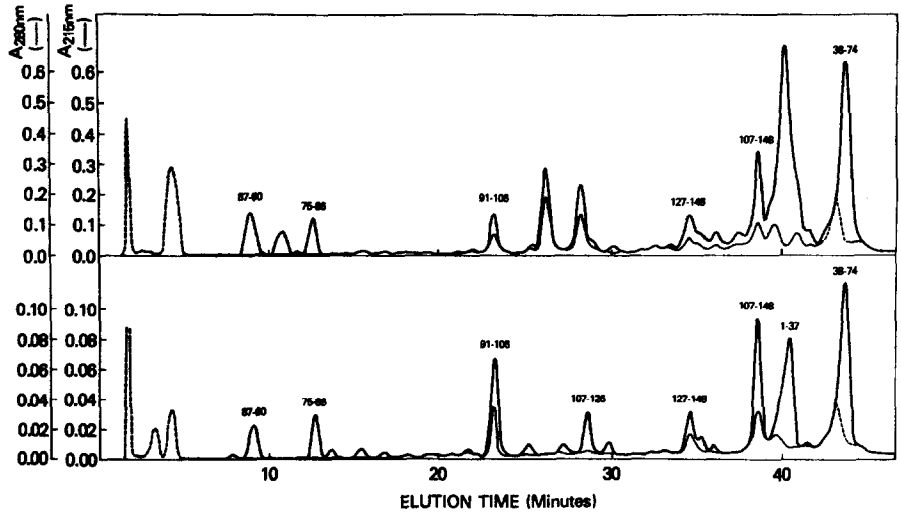


Fig. 5. HPLC peptide map of iodinated calmodulin. Clostripain digests of calmodulin (50 μg, bottom panel) and iodinated calmodulin (450 μg, top panel) were resolved on a μBondapak $C_{18}$ column, as described in the text. The unmodified peptides can be identified by their characteristic retention times. In the digest of iodinated calmodulin, the appearance of new peaks of absorbance at 280 nm eluted at 25.9, 38.0, 39.3, and 40.6 min, indicate mono- and di-iodination of Tyr residues 99 and 138, resulting in delayed elution of peptides 91–106 and 107–148.

peaks of radioactivity, eluted at 40 min, appear to reflect incompletely digested material, since they were not observed in other digests of [$^3$H]calmodulin.

Iodinated calmodulin, prepared by a glucose oxidase–lactoperoxidase method, has also been characterized by this peptide mapping procedure (Fig. 5). The appearance of peaks of absorbance at 280 nm, eluted at 25.9, 28.0, 39.3, and 40.6 min, suggests mono- and di-iodination of both Tyr residues 99 and 138. Identification of the peptides at 25.9 and 28.0 as modified 91–106 was confirmed by amino acid analysis (data not shown). The persistence of peaks at 23.1 and 38.3 min, corresponding to unmodified peptides 91–106 and 107–148, indicates that iodination of Tyr-99 and Tyr-138 was incomplete.

## DISCUSSION

In recent years, reversed-phase HPLC has been widely employed in the resolution of a variety of proteins and peptides. In the case of calmodulin, the alkylphenyl-bonded phase has proven useful for separation of calmodulin from homologous $Ca^{2+}$-binding proteins[28,32]. The use of a mobile phase containing 0.01 *M* phosphate buffer (pH 6.0)[33] facilitates elution of calmodulin at neutral pH with relatively low concentrations of acetonitrile, allowing recovery of calmodulin in a biologically active state. The retention of calmodulin on the alkylphenyl column has been shown to be decreased in the presence of contaminating $Ca^{2+}$ in the elution buffer[28]. In this case, the elution of calmodulin occurs as a double peak, corresponding to the calcium-complexed and calcium-free states. Because addition of calcium to the phos-

phate buffer induces precipitation, EGTA is included in the mobile phase to insure homogeneous elution of calmodulin in the calcium-free state[28,32].

The development of a two-step procedure, consisting of CAPP–Affigel affinity chromatography, followed by HPLC on an alkylphenyl column, allows isolation of calmodulin from cell extracts and complex incubation mixtures. The use of reversed-phase HPLC as a final step in the purification provides several advantages, including high resolution and excellent recovery (80–90% from samples ranging from 35 μg to 3 mg of protein)[28,32]. Thus, this HPLC step is ideal for the isolation of radiolabeled calmodulin derivatives on a microscale.

Reversed-phase HPLC on an alkylphenyl column has previously been employed as a rapid and efficient method for analyzing tryptic digests of calmodulin derived from several species[20,34,35]. Rapid peptide mapping of calmodulin has also been performed on a macroreticular anion-exchange resin[36]. For the present analysis, a $C_{18}$ column, which exhibits greater hydrophobic character than the alkylphenyl column, has been employed for the separation of small calmodulin peptides. The use of phosphoric acid, a hydrophilic ion-pairing solvent, has been shown to facilitate elution of underivatized peptides from the $C_{18}$ column at significantly lower concentrations of acetonitrile[33]. Using this approach, Fullmer and Wasserman[26] have described the analytical peptide mapping of intestinal calcium-binding protein on a $C_{18}$ column eluted with a linear gradient from 0.1% phosphoric acid to acetonitrile. This method also provides an efficient, highly reproducible separation of calmodulin peptides, derived by digestion with clostripain. Under these elution conditions, retention of the peptides is highly sensitive to chemical modification. Oxidation of methionyl residues with performic acid decreases the retention times of the modified peptides, whereas iodination of tyrosines or guanidination of lysines increases retention times of the modified peptides. In each case, even a single chemical modification results in a detectable change in retention time, allowing a preliminary assessment of the intramolecular distribution of modifications by examination of the HPLC elution profile.

Previous reports of HPLC peptide mapping of calmodulin have involved resolution of peptides, generated by digestion with trypsin[20,34–36]. Clostripain, a sulfhydryl protease from *Clostridium histolyticum*, possesses several advantages over trypsin for reproducible peptide mapping of calmodulin and its modified derivatives. First, as previously reported[29], clostripain exhibits specificity for arginyl residues, whereas trypsin cleaves at both lysyl and arginyl residues. Thus, the pattern of digestion of calmodulin with clostripain is insensitive to guanidination of lysyl residues, as well as to oxidation of methionines or iodination of tyrosines. Second, trypsin induced cleavage of the peptide bonds between Met-71 and Met-72 and following the two methionines at 144,145, which was previously encountered in the process of sequencing of calmodulin[37], is not observed with clostripain. Thus, the specificity and reproducibility of clostripain digestion facilitates the interpretation of the calmodulin peptide maps.

The HPLC procedures for isolation and peptide mapping of calmodulin and its derivatives are applicable to analysis of the structural–functional relationships of calmodulin. These methods have been used to study the role of lysyl residues in the interaction of calmodulin with its target proteins[27]. A modification of these methods has also been developed to quantitate covalent attachment of norchlorpromazine-

isothiocyanate to calmodulin[17]. Application of these methods to the identification of specific modifications of calmodulin should facilitate assessment of the role of individual amino acid residues in determining the physical, chemical, and spectroscopic properties of this unique calcium-binding protein.

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