Affinity Selection of Chemically Modified Proteins: Role of Lysyl Residues in the Binding of Calmodulin to Calcineurin

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ABSTRACT: In affinity selection, calcineurin selects from a population of randomly modified calmodulins those species with which it prefers to interact. The method shows that acetylation of lysines affects calmodulin so as to interfere with its ability to interact with calcineurin. Monoacetylation of any lysine of calmodulin reduces its affinity for calcineurin by 5–10-fold. Multiple acetylations amplify the loss of affinity; none of the modifications are incompatible with activity. The lack of selectivity of calcineurin against any particular modified lysine indicates that the loss of affinity reflects changes induced by the removal of the charged groups and suggests an important role for electrostatic interactions in the cooperative structural transitions which calmodulin undergoes upon binding its target proteins or calcium. In the presence of calcineurin, a large and specific decrease in the rate of acetylation of Lys-75 and -148 of calmodulin is observed. The reactivity of the same residues is greatly increased in the presence of calcium alone [Giedroc, D. P., Sinha, S. K., Brew, K., & Puett, D. (1985) *J. Biol. Chem. 260*, 13406–13413]. Lys-75, located in the central helix, and the C-terminal Lys-148 [Babu, Y. S., Sacks, J. S., Greenhouse, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature (London) 315*, 37–40] may act as sensors of the calmodulin allosteric transitions. Their reactivity changes in opposite directions in response to calcium-induced or calcineurin-induced structural changes. The reactivity of other residues such as Lys-21, decreased in the presence of calcineurin but not calcium, is also affected by a conformational change which is induced specifically by calcineurin.

Calmodulin, a member of a family of homologous calcium binding proteins, is unique because of its ability to interact with a large number of proteins, peptides, and drugs [reviewed by Klee & Vanaman (1982)]. The structural features which account for calmodulin's interaction with so many different molecules are not yet clearly defined. In most cases, the calmodulin targets interact only with the calmodulin–calcium complex, but at least four proteins, phosphorylase kinase (Cohen et al., 1978), the M_r 110000 brush border protein (Glenney & Weber, 1980), bacterial adenylate cyclase (Greenlee et al., 1982), and a brain protein isolated by Andreasen et al. (1983), bind to the calcium-free conformer. The structural requirements of different enzymes for interaction with and activation by calmodulin are different (Thiry et al., 1980; Newton et al., 1984, 1985; Putkey et al., 1986). Some enzymes, such as phosphorylase kinase (Kuznicki et al., 1981) and the Ca^{2+} + Mg^{2+} plasma membrane ATPase (Guerini et al., 1984), are activated by calmodulin fragments. Others, such as phosphodiesterase, calcineurin, and myosin kinase, interact with but are not activated by the COOH-terminal fragment 78–148 (Newton et al., 1984; Ni & Klee, 1985). A one to one, covalent, adduct of calmodulin with norchlorpromazine isothiocyanate, $CAPP_1$–calmodulin, can still bind all calmodulin-regulated enzymes with high affinity, suggesting that at least some differences exist between the enzyme and the phenothiazine binding sites (Newton et al., 1985).

Modifications of specific amino acid residues of calmodulin have different effects on the ability of calmodulin to activate enzymes. For example, carbamoylation of the lysines decreases the affinity of calmodulin for phosphodiesterase without affecting its activation of adenylate cyclase (Thiry et al., 1980). In the present study, we examine the role of lysyl residues of calmodulin in its interaction with calcineurin. In addition to studying the effect of calcineurin on the rate of acetylation of individual lysyl residues, we use affinity selection, a technique whereby calcineurin is used to select from a population of randomly modified calmodulins those species with which it prefers to interact. Comparison of the pattern of modification of individual lysyl residues in those calmodulin molecules which bind to calcineurin with that of those rejected suggests that the overall charge of the molecule plays a predominant role in governing its interaction with calcineurin.

Experimental Procedures

Materials. Calcineurin was purified from bovine brain [as previously described in Klee et al. (1983)]. Calmodulin from bovine testes was prepared by modification of the methods of Autric et al. (1980) and Klee (1977). Dephosphorylated chicken gizzard smooth muscle myosin light chains, prepared by the method of Hathaway and Haeberle (1983), and myosin kinase, prepared as described (Adelstein & Klee, 1981), were generously provided by Dr. David R. Hathaway, Indiana University School of Medicine. The catalytic subunit of cAMP-dependent protein kinase, prepared by the method of Peters et al. (1977), was the generous gift of Dr. Edmond Fischer, University of Washington, Seattle. ^{32}P-Phosphorylated myosin light chains and ^{32}P-phosphorylated histone V were prepared as previously described (Klee et al., 1983). Clostripain was purchased from Boehringer Mannheim. Histone type VS and N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK)[1] were from Sigma Chemical Co. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was from Worthington. *O*-Methylisourea was purchased from

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[1] Abbreviations: TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; RT, retention time; CaM, calmodulin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol.

Nutritional Biochemicals Corp. [^{3}H]Acetic anhydride (9 Ci/mmol) and [1-^{14}C]acetic anhydride (115 mCi/mmol) were from Amersham. Fluorescamine was a product of Roche Laboratories.

Chemical Modification of Lysyl Residues of Calmodulin. (*A*) *Guanidination.* Guanidination of calmodulin was performed with *O*-methylisourea by a procedure originally described for guanidination of ribonuclease (Klee & Richards, 1957). Calmodulin (15.3 mg) was dissolved in 3.1 mL of 0.5 M *O*-methylisourea–HCl at pH 10.5 and incubated for 25 h at 2 °C. The reaction was terminated by gel filtration on a 1.5 × 17 cm Sephadex G-10 column equilibrated and eluted with 0.05 M ammonium bicarbonate. The guanidinated protein contained 2.0 lysines and 5.4 homoarginines/mol.

(*B*) *Acetylation of Calmodulin.* (a) Complete acetylation with acetic anhydride was performed at 0 °C by the method of Fraenkel-Conrat (1957). Five 2-μL aliquots of acetic anhydride were added at 12-min intervals to a 0.2-mL solution of calmodulin (28.8 mg/mL) in 50% saturated sodium acetate. The course of the reaction was followed by assay of primary amino groups. After 60-min incubation, the acetylated protein was isolated by gel filtration on a 9-mL Sephadex G-25 column equilibrated and eluted with 0.05 M ammonium bicarbonate. Fractions containing protein were pooled, lyophilized, and dissolved in 0.8 mL of 0.04 M Tris-HCl buffer, pH 8, containing 0.1 M NaCl. Deacetylation of *O*-acetyltyrosine was performed for 30 min at 22 °C as described by Richman (1979). Acetylated calmodulin was finally isolated by gel filtration on Sephadex G-25 as above. Under these conditions, 6.6 of the 7 lysyl residues of calmodulin were acetylated.

(b) Limited acetylation of calmodulin with radiolabeled acetic anhydride was performed in a fume hood. In method 1, 3.8 mg of calmodulin in 200 μL of 50% saturated sodium acetate was injected into a vial containing 100 mCi of [^{3}H]-acetic anhydride (1.1 mg) and 2 μL of unlabeled acetic anhydride which had been previously cooled in dry ice. The vial was sealed and transferred to an ice bath. The mixture was incubated at 0 °C for 30 min with periodic agitation. A more limited acetylation was obtained by using 500 μCi (0.46 mg) of [1-^{14}C]acetic anhydride followed by a 60-min incubation at 0 °C. For method 2, acetylation was performed at 0 °C by injection of a solution of calmodulin (5.2 mg in 1 mL of 0.04 M Tris-HCl, pH 8, 0.2 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) into a vial containing 25 mCi (0.34 mg) of [^{3}H]acetic anhydride (7.48 Ci/mmol). After 2 min, 25 μL of 3.8 M hydroxylamine was added. Gel filtration and hydroxylamine treatment (method 1) of radiolabeled calmodulin were performed as described above. All chemically modified proteins were lyophilized and stored at −70 °C.

(*C*) *^{14}C-Acetylation of Calmodulin in the Absence or Presence of Calcineurin.* Reaction mixtures (3.1 mL) contained 20 mM Tris-HCl, pH 8, 0.2 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM dithiothreitol, and 5.77 μM calmodulin with or without calcineurin. [^{14}C]Acetic anhydride (2.75 μmol, 0.314 mCi) in 50 μL of acetonitrile was added to each reaction mixture. After 30 min at 0 °C, excess reagent, calcineurin, and calmodulin were resolved by gel filtration on identical Sephadex G-200 superfine columns (1.5 × 26.5 cm) equilibrated and eluted with 0.04 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM $MgCl_2$, 2 mM EGTA, and 0.5 mM dithiothreitol. The flow rate was 3 mL/h and the fraction size 1 mL. The calmodulin was purified by HPLC as described below.

^{3}H-Acetylated Calmodulin Binding to Calcineurin. ^{3}H-Acetylated calmodulin (0.35 mol of acetyl/mol of calmodulin), 1.6 μM, was incubated at 0 °C for 90 min with 1.0 μM calcineurin in 0.05 M Tris-HCl, pH 8.0, containing 0.05 M NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM dithiothreitol, 2 μg/mL soybean trypsin inhibitor, and 1 μg/mL leupeptin (total volume 0.8 mL). To separate the bound from free ^{3}H-acetylated calmodulin, 200-μL aliquots of the incubation mixture were layered on top of 10–40% (v/v) glycerol gradients made in the same buffer (3.6 mL), resting on 0.1-mL cushions of 75% glycerol. Centrifugation was at 55 000 rpm at 2 °C for 21.5 h in a Beckman SW 60 rotor. Fractions (0.13 mL) were collected from the bottom of the tubes. Fractions containing free calmodulin and fractions containing calmodulin bound to calcineurin were purified by HPLC as described below. Recoveries of ^{3}H-acetylated calmodulin were 55–75%.

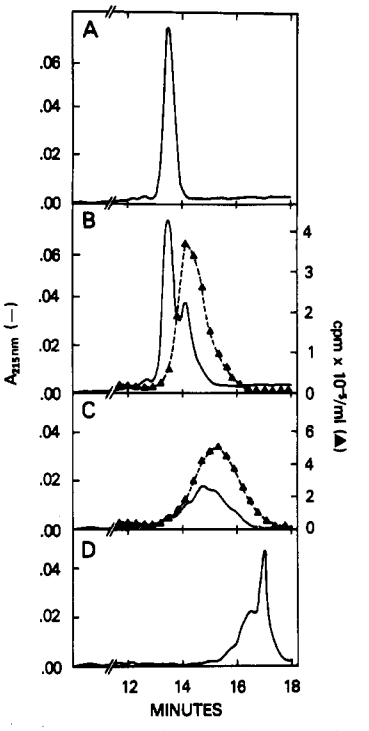

FIGURE 1: Resolution of calmodulin and its acetylated derivatives by reverse-phase HPLC on a C_{18} μBondapak column. (A) Calmodulin, 10 μg; (B) ^{3}H-acetylated calmodulin, 13 μg (0.35 mol of acetyllysine/mol); (C) ^{3}H-acetylated calmodulin, 10 μg (2.5 mol of acetyllysine/mol); and (D) acetylated calmodulin, 11 μg (6.6 mol of acetyllysine/mol).

Purification of Radiolabeled Calmodulin by HPLC. Acetylated calmodulin was purified on a μBondapak phenyl reverse-phase column (Waters Associates) by a modification of published procedures (Klee et al., 1981; Manalan et al., 1985). After gel filtration, ^{14}C-acetylated calmodulin was loaded onto the column equilibrated with 0.01 M potassium phosphate buffer, pH 6.0, containing 10 μM $CaCl_2$ (buffer A) and eluted with a 10-min linear 0–15% acetonitrile gradient in buffer A followed by a 20-min linear 15–35% acetonitrile gradient in the same solvent at a flow rate of 1.5 mL/min. ^{3}H-Acetylated calmodulin from glycerol gradients was applied to the column equilibrated with 85% buffer B (0.01 M potassium phosphate buffer, pH 6.0, containing 1 mM EGTA) and 15% acetonitrile. Calmodulin was eluted with a 20-min, 15–35%, linear gradient of acetonitrile in buffer B at a flow rate of 1.5 mL/min. Calmodulin, not resolved from its acetylated derivatives, eluted as a single peak (RT = 19 min). Fractions were collected in polyethylene tubes containing 100 μg of calmodulin as carrier. The specific activities of bound and free calmodulin were calculated from the elution profile at 215 nm calibrated with a standard curve. The protein was immediately freed of HPLC solvents by gel filtration on

Pharmacia PD10 columns equilibrated in the appropriate buffers.

Characterization of Acetylated Calmodulin by HPLC. Calmodulin and acetylated calmodulins were resolved on a C_{18} μBondapak reverse-phase column (3.9 mm × 30 cm, 10-μm beads, Waters Associates) using a 20 min, 30–60%, linear gradient of acetonitrile in solvent C [0.1% (v/v) orthophosphoric acid, pH 2.2]. The flow rate was 1.5 mL/min. Calmodulin eluted as a single symmetrical peak at 13.4 min (Figure 1, panel A), whereas highly acetylated calmodulin (average of 6.6/7 lysyl residues modified) eluted as overlapping peaks with retention times between 15 and 17 min (Figure 1, panel D). Partially acetylated calmodulin (2.5/7 lysyl residues modified) eluted as a broad peak with retention times between 13.4 and 16.5 min (Figure 1, panel C). Calmodulin with only 0.35 mol of acetyllysine/mol eluted as two major peaks (Figure 1, panel B). The first peak, devoid of radioactivity, was at the position of calmodulin. A second, asymmetric, peak was associated with the bulk of radioactivity and eluted at 14.1 min. This material, highly enriched in monoacetylated calmodulin, was resolved from unlabeled calmodulin and from more extensively substituted calmodulin, eluting later, by a second HPLC.[2] These results show that the calculated stoichiometry of lysyl modification is representative of the actual stoichiometry of calmodulin modification, rather than of the average of a bimodal distribution of largely unmodified and fully acetylated calmodulin molecules.

Peptide Mapping. (*A*) *Proteolytic Digests*: (*a*) *Clostripain.* Clostripain was selected to prepare digests which would be unaffected by covalent modification of lysyl residues. Clostripain (6.6 mg/mL in 0.2 M sodium phosphate buffer, pH 7.8) was activated by overnight treatment with 10 mM dithiothreitol at 0–4 °C (Porter et al., 1971). Calmodulin or acetylated calmodulin was dissolved in 0.05 M sodium phosphate buffer, pH 7.8, containing 2 M urea, 1 mM $CaCl_2$, and 0.5 mM dithiothreitol, at concentrations of 15–90 μM. Clostripain was added at 0.11 mg/mL (2.2 μM), and the digestion mixture was incubated at 37 °C for 3 h. A second (equal) aliquot of clostripain was added, and digestion at 37 °C was continued for an additional 3 h. Digestion was stopped by the addition of TLCK (final concentration 10 μM). (*b*) *Trypsin.* Calmodulin or acetylated calmodulin was dissolved in 0.05 M ammonium bicarbonate containing 0.06% thiodiglycol and 4 mM EGTA at 1.3–2.0 mg/mL. After 10 min at 37 °C, trypsin (1 mg/mL in 0.001 N HCl) was added to achieve a concentration of 0.2 mg/mL, and the incubation was continued for 60 min at 37 °C. A second identical aliquot of trypsin was then added and digestion continued for an additional 60 min. Digestion mixtures were stored at −70 °C.

(*B*) *Peptide Mapping by HPLC.* Calmodulin peptides were separated, using a μBondapak C_{18} reversed-phase column (3.9 mm × 30 cm, 10-μm beads, Waters Associates), by a modification (Manalan et al., 1985) of the method of Fullmer and Wasserman (1979). Clostripain and tryptic peptides were separated with a 60 min, 0–60%, linear gradient of acetonitrile in solvent C at flow rates of 2 and 1.5 mL/min, respectively. When needed, tryptic peptides, diluted with an equal volume of H_2O, were rechromatographed on the same column using gradients of acetonitrile, as indicated in legend to Figure 3, in 0.1% (v/v) trifluoroacetic acid over 60 min at a flow rate of 1.5 mL/min. Peptides were identified from their amino acid composition.[3]

The peptides generated by clostripain (Figure 2, lower panel) corresponded to specific cleavages at the carboxyl side of arginyl residues, a finding consistent with the specificity of clostripain (Mitchell & Harrington, 1968). The peptide bond between arginine-126 and glutamic acid-127 was relatively resistant to proteolysis. Even upon extensive digestion, only limited cleavage of peptide 107–148 was observed (Figure 2). Acetylated peptides elute later than the corresponding peptides of unmodified calmodulin (Figure 2, upper panel). Peptides 91–106, 127–148, and 107–148, each of which possesses a single acetylated lysyl residue, eluted 1–3 min later than did the unmodified peptides. Acetylated peptide 75–86, which contains two lysyl residues, eluted in two distinct peaks, at 15 and 18 min, representing peptides with modification of one or two lysyl residues, respectively, as described below. Acetylated peptide 1–37, containing three lysyl residues, eluted at 44 min. Complete digestion of fully acetylated calmodulin with clostripain was never achieved. A cluster of incompletely digested acetylated peptides corresponding to calmodulin peptide 1–86[3] eluted between 50 and 53 min. The low recovery of peptides 1–37, 38–74, and 75–86 is consistent with this identification. Undigested peptide 1–86 was also detectable in peptide maps of partially acetylated calmodulin (Figure 2, middle panel). Partially acetylated calmodulin (2.8 acetyllysines/mol of calmodulin) displayed an intermediate elution profile reflecting the presence of unmodified, as well as partially and fully acetylated, peptides. Unmodified peptide 75–86 eluted at 13 min. Mono- and diacetylated peptides 75–86 identified by their specific activities eluted at 15 or at 18 min, respectively. Peptide 91–106 (RT = 26 min) was completely acetylated and represented 35% of the total radioactivity incorporated into calmodulin. Under more limiting conditions of acetylation (0.29 mol of acetyllysine/mol of calmodulin), over 60% of the radiolabel incorporated was associated with Lys-94. Low levels of acetylation were detected in all the other lysine-containing peptides. The distribution of the radiolabel documents the strong preference of the reaction of acetic anhydride in sodium acetate for Lys-94. Treatment of calmodulin with acetic anhydride in 0.04 M Tris-HCl, pH 8.0, 0.2 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ reduced the specificity of the acetylation reaction for Lys-94 and favored acetylation of other lysyl residues, notably Lys-75 (Table I).

Digestion with trypsin was used to further define the distribution of acetylated lysines (Figure 3). Peptide bonds between Lys-21 and Asp-22, and between Lys-94 and Asp-95, are resistant to trypsin (Watterson et al., 1980). Because acetylated lysyl residues are also resistant to trypsin cleavage, the pattern of the tryptic digest of highly acetylated calmodulin resembled that of the clostripain digests (Figure 3, top panel). Partially acetylated calmodulin (Figure 3, middle panel) yields additional labeled peptides due to the uneven distribution of acetylated lysines. Peptide 1–37 which contains three lysines at positions 13, 21, and 30 (Lys-21 is resistant to trypsin) can produce at most seven labeled peptides (14–30, mono- and diacetylated 1–30 and 14–37, and di- or triacetylated 1–37) depending upon the extent of acetylation of the three lysyl residues. A small radioactive peptide, which could not be

[2] Radiolabeled acetylated calmodulin (0.5 mg, 0.35 mol of acetyllysine/mol) was applied to a C-18 column and eluted with a 30–60% gradient of acetonitrile in 0.1% (v/v) H_3PO_4 over 30 min. The absorbance at 215 nm and the radioactivity were monitored. The distribution of calmodulin derivatives was 67% unlabeled, 31% monoacetylated, and less than 3% multiply acetylated calmodulin. Rechromatography on the same column of the monoacetylated species yielded a homogeneous protein containing 0.8 mol of acetyllysine/mol (measured by radioactivity). Monoacetylated calmodulin and unlabeled calmodulin (eluted prior to the labeled protein on the first column) activated calcineurin with $K_{0.5}$ values of 2×10^{-8} and 2×10^{-9} M, respectively.

[3] Amino acid analyses of peptides were provided to the reviewers.

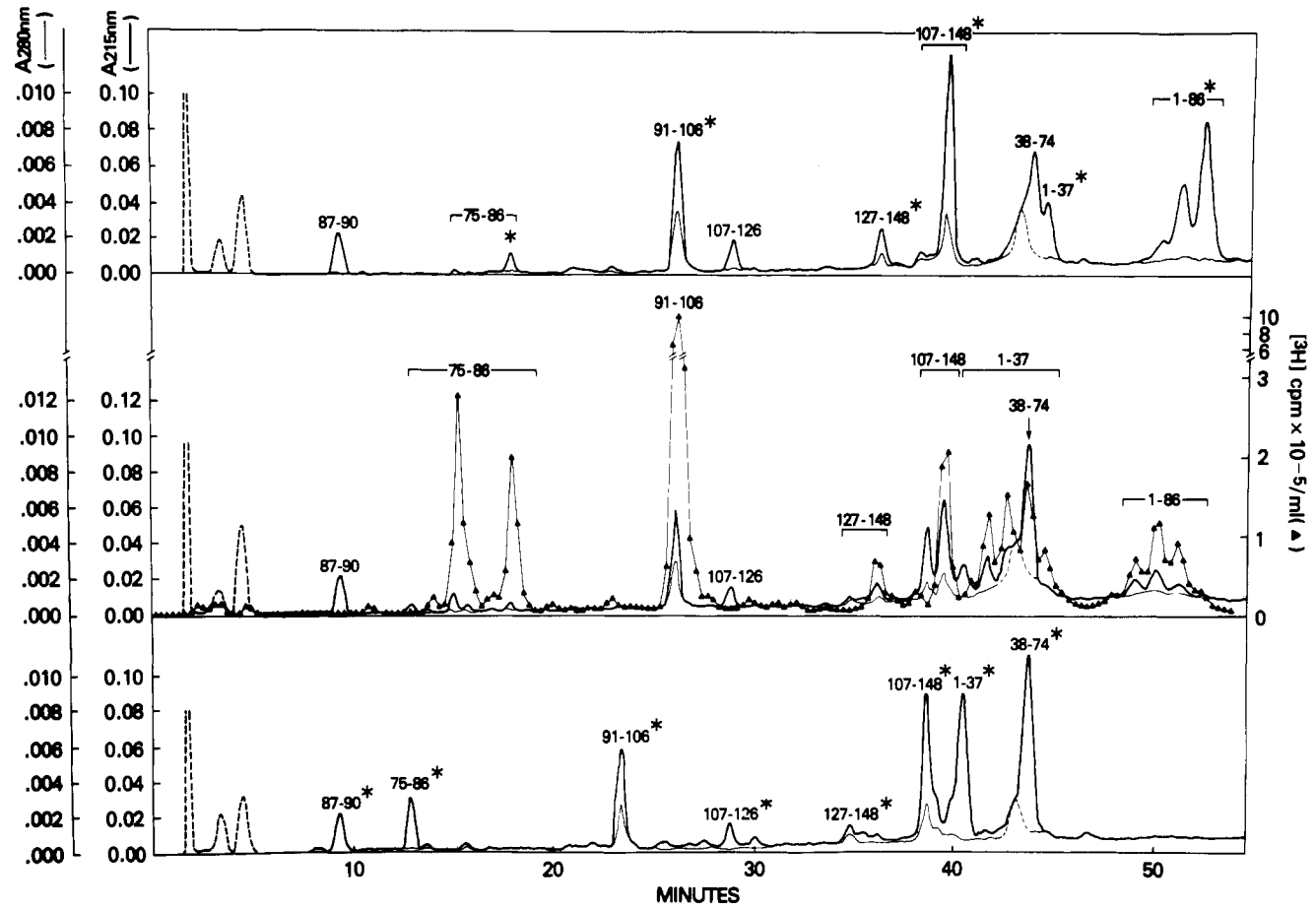

FIGURE 2: HPLC elution profiles of clostripain digests of calmodulin (80 μg, bottom panel), ^{3}H-acetylated calmodulin (80 μg, 2.8 mol of acetyllysine/mol, middle panel), and acetylated calmodulin (80 μg, 6.6 mol of acetyllysine/mol, top panel). Identification of peptides was based on amino acid analysis (asterisk) or retention times. Broken lines indicate UV-absorbing peaks contributed by additional reagents in digestion mixtures or solvents.

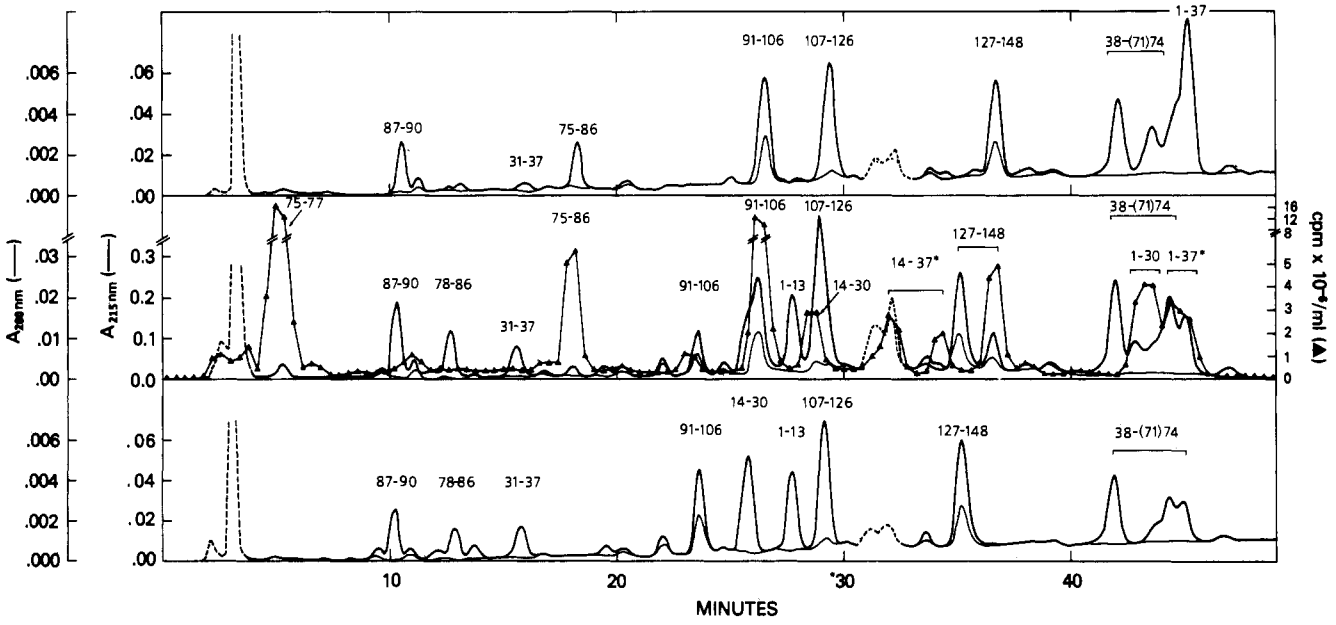

FIGURE 3: HPLC peptide maps of tryptic digests of calmodulin (52 μg, bottom panel), ^{3}H-acetylated calmodulin (400 μg, 2.5 mol of acetyllysine/mol, middle panel), and acetylated calmodulin (46 μg, 6.6 mol of acetyllysine/mol, top panel). Calmodulin peptides were identified by their retention times determined by Newton and Klee (manuscript in preparation). Fully acetylated peptides 75–86, 91–106, 127–148, and 1–37 have the same retention times as the corresponding clostripain peptides identified in Figure 2 (top panel). Peptide 75–77 was identified, by its amino acid composition, without further purification. Acetylated peptides 14–30, 14–37, and 1–30, obtained by a 300-min digestion with trypsin (2 μg/mL), were isolated by two successive HPLC steps, using (1) a 0–50% gradient of acetonitrile in solvent C over 60 min and (2) acetonitrile gradients in 0.1% fluoroacetic acid [15–50% over 60 min (peptide 14–30), 20–40% over 60 min (peptide 14–37), and 20–50% over 70 min (peptide 1–30)]. The peptides not definitively identified are shown by the asterisks.

detected by its absorbance, eluted at 5 min and was identified as Lys-Met-Lys by amino acid analysis. Unlike the experience of Giedroc et al. (1985), no chymotryptic cleavages were observed under our conditions.

Table I: Effect of Calcineurin on Acetylation of Calmodulin[a]

	calmodulin		bound:free reactivity ratio
	+calcineurin	−calcineurin	
CaM sp act. (cpm/nmol)	91 300	125 000	0.73
clostripain peptides (cpm)[b]			
1–37 (13, 21, 30)[c]	57 700	48 700	1.18
75–86a (75 or 77)	25 300	55 900	0.45
75–86b (75 + 77)	3 600	15 700	0.23
1–86 (13, 21, 30, 75, 77)	8 600	48 200	0.18
91–106 (94)	58 800	52 000	1.12
107–148 + 127–148 (148)	8 100	14 600	0.54
trypsin peptides (cpm)			
14–30 (21)	9 950	13 200	0.72
14–37 (30)[d]	19 200	14 200	1.35
14–37 (21 + 30)[d]	4 300	9 900	0.44
1–30 (13)	17 700	15 500	1.14
1–30, 1–37 (13, 21, 30)[d]	19 000	30 200	0.63
75–77 (75)[e]	16 800	65 100	0.26
75–86a (77)[e]	16 700	9 700	1.72
75–86b (75 + 77)[e]	4 400	18 700	0.24
91–106 (94)	63 900	54 700	1.17
127–148 (148)	8 300	16 000	0.52

[a] Calmodulin, acetylated with [^{14}C]acetic anhydride in the free state or in the presence of 1 molar equiv of calcineurin, was isolated as described under Experimental Procedures. The distribution of acetylated lysyl residues was determined by HPLC peptide mapping after digestion with clostripain or trypsin using the retention times shown in Figures 2 and 3. [b] The same amount of calmodulin was subjected to proteolysis and HPLC. The cpm associated with each peptide was corrected for recovery of radioactivity after HPLC: 95% and 92% for the clostripain and trypsin digests of free calmodulin, respectively, and 93% and 100% for the corresponding digests of calmodulin labeled in the presence of calcineurin. [c] Numbers in parentheses indicate acetylated lysyl residues in corresponding peptides. [d] These peptides have only been tentatively identified. [e] According to these assignments, the reactivity ratio of Lys-75 is 0.39 and that of Lys-77, 1.0.

Protein Phosphatase Assay. Phosphatase activity was assayed in 50 or 100 μL of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 10 μM $MnCl_2$, 0.5 mM dithiothreitol, 10^{-9} M calcineurin, and either 1 μM ^{32}P-phosphorylated myosin light chains or 5 μM ^{32}P-phosphorylated histone V in the presence of $CaCl_2$ (1 mM) or EGTA (0.2 mM) at 30 °C for 30 min. Acetylated (6.6 mol of acetyllysine/mol), guanidinated (5.4 mol of homoarginine/mol), or unmodified calmodulin was included in assay mixtures as indicated. After addition of 0.5 mL of 0.1 M potassium phosphate, pH 7.0, containing 5% (w/v) trichloroacetic acid, inorganic phosphate was isolated by passage through 0.5-mL columns of Dowex AG 50-X8, H^+ form, as described (Manalan & Klee, 1983). The specific activity of the enzyme, in the presence and absence of calmodulin, was 10 and 2 nmol min^{-1} mg^{-1}, respectively.

Phosphodiesterase Assay. Phosphodiesterase activity was measured as previously described (Klee, 1977). The assay mixture contained 0.04 M Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 0.2 M NH_4Cl, 0.1 mM DTT, 1 mM cAMP, [^{3}H]cAMP (60 000–100 000 cpm), [^{14}C]AMP (2000–2500 cpm), and phosphodiesterase in a final volume of 0.1 mL. Activity was assayed in the presence of 0.05 mM EGTA (basal) or of 0.05 mM $CaCl_2$.

Quantitative Analysis of Amino Groups. Amino groups were quantitated with fluorescamine by a modification of the methods of Udenfriend et al. (1972) and Lai (1977). Aliquots (5 μL) were withdrawn at 12-min intervals and added to 1.5 mL of 0.2 M borate (Na) buffer, pH 9.0. Fluorescamine (0.5 mL of 20 mg/100 mL of acetone) was added to each tube while the contents were continuously mixed with a Vortex mixer for 5 s. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co.) using an excitation wavelength of 390 nm and an emission wavelength of 475 nm. Assays were calibrated from a standard curve using unmodified calmodulin. A linear response was obtained in the range of 5–50 nmol of lysyl residues. The color factor exhibited by lysyl residues of calmodulin is similar to that of N^{α}-acetyl-L-lysine.

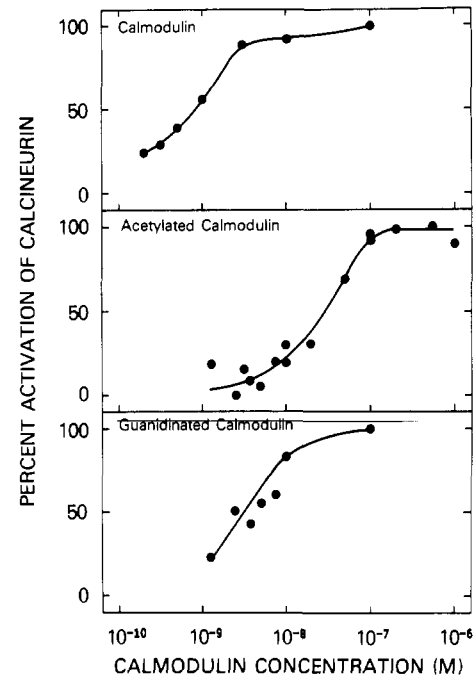

FIGURE 4: Activation of calcineurin by calmodulin and its chemically modified derivatives. Phosphatase activity was assayed by using ^{32}P-phosphorylated histone VS as a substrate. The concentration of calcineurin in the assays was 10^{-9} M.

Protein concentration was measured spectroscopically by using the extinction coefficients $\epsilon^{1\%}_{277nm} = 9.26$ for calcineurin (Klee et al., 1983) and $\epsilon^{1\%}_{277nm} = 1.8$ for calmodulin (Richman & Klee, 1979) or by amino acid analysis. The latter was performed by a modification of the method of Spackman et al. (1958) as described by Newton et al. (1984).

Results

Two complementary approaches were used to study sites on calmodulin involved in interaction with calcineurin. We determined the effect of calcineurin binding on the susceptibility of calmodulin to acetylation and correlated these results with effects of acetylation of calmodulin on its ability to bind to calcineurin. To discriminate among the seven lysyl residues in calmodulin, a broad distribution and low level of modification were required.

Effect of Modification of Lysyl Residues of Calmodulin on Its Affinity for Calcineurin. Calmodulin stimulates the dephosphorylation of ^{32}P-histones by calcineurin with high affinity (Figure 4, upper panel). Since the concentration of calcineurin in the assays was 1 nM, the $K_{0.5}$ for activation of 0.9 nM is an overestimate of the K_{diss} for calmodulin binding to the enzyme. Guanidination of 5.4 of the 7 lysyl residues of calmodulin produced only a small increase in the $K_{0.5}$ to 3 nM calmodulin (Figure 4, lower panel). Acetylation of 6.6 of the 7 lysyl residues of calmodulin resulted in a greater increase in $K_{0.5}$ to 25 nM (Figure 4, center panel). With either guanidinated or acetylated calmodulin, the maximal activation of calcineurin was the same as that observed with native calmodulin (similar results were obtained by using ^{32}P-phosphorylated protein phosphatase inhibitor 1 as a substrate).

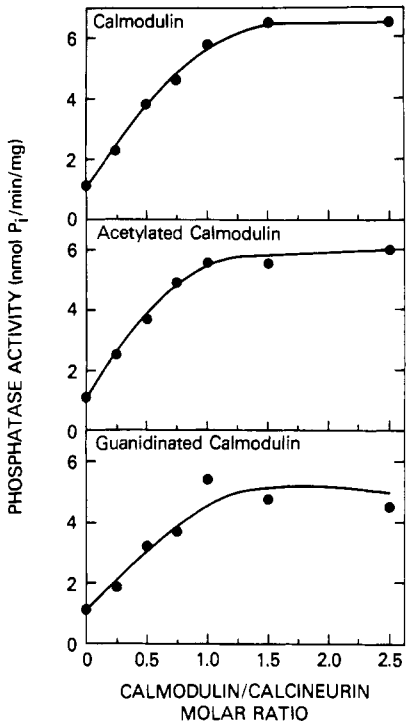

FIGURE 5: Titration of calcineurin with calmodulin and its chemically modified derivatives. The substrate was ^{32}P-phosphorylated smooth muscle myosin light chains. Calcineurin concentration was 0.2 μM.

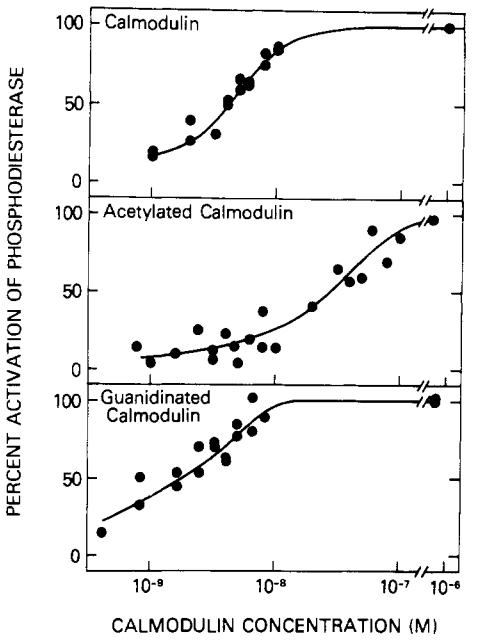

FIGURE 6: Activation of cyclic nucleotide phosphodiesterase by calmodulin and its chemically modified derivatives. Phosphodiesterase concentration was 40 pM.

Since at least 80% of the lysyl residues have been modified, these results suggest a decrease in affinity rather than loss of the ability of a significant fraction of the calmodulin to activate calcineurin as a result of the chemical modifications. To examine this point more directly, the phosphatase activity of calcineurin was measured at a high concentration (0.2 μM) of calcineurin. Calcineurin was stimulated 6-fold by calmodulin or by its acetylated or guanidinated derivatives (Figure 5). In each case, maximal stimulation of phosphatase activity was achieved at a calmodulin:calcineurin ratio of 1:1, excluding inactivation of a significant fraction of calmodulin by lysyl modification as an explanation for the changes in activation constants. Thus, the covalent modification of the lysyl residues of the protein results in a decrease in affinity of calmodulin for calcineurin. Because the effect of acetylation is much greater than that of guanidination, acetylation of calmodulin was selected for subsequent experiments.

For purposes of comparison, effects of lysyl modification on calmodulin activation of cyclic nucleotide phosphodiesterase are presented in Figure 6. Unmodified calmodulin stimulated phosphodiesterase activity 7-fold, with half-maximal activation at 4 nM calmodulin (Figure 6, upper panel). Guanidinated calmodulin (5.4 mol/mol) also stimulated phosphodiesterase activity 7-fold, with half-maximal activation at approximately 2 nM. Thus, although guanidination of calmodulin slightly reduced its affinity for calcineurin, no such reduced affinity was observed with phosphodiesterase. Acetylated calmodulin (6.6 mol/mol) stimulated phosphodiesterase 7-fold, with half-maximal activation at 26 nM acetylated calmodulin. The decrease in affinity was smaller than that seen with calcineurin.

Effect of Calcineurin on Acetylation of Calmodulin. Calmodulin was acetylated either in the free state or as a 1:1 complex with calcineurin. The extent of acetylation of calmodulin was reduced by 27% in the presence of calcineurin (Table I). Specific activities were 125 000 cpm/nmol for calmodulin acetylated in the free state and 91 300 cpm/nmol for calmodulin acetylated in the presence of calcineurin. These specific activities correspond to 0.98 and 0.72 mol of acetyl groups incorporated/mol of calmodulin, respectively. The reduced level of acetylation of calmodulin observed in the presence of calcineurin is unlikely to be due to depletion of reagent by reaction with calcineurin since, as described below, the inhibition of acetylation selectively affects specific lysyl residues.

As shown by the distribution of radioactivity in the clostripain peptides (Table I), calcineurin inhibited the acetylation of Lys-75 and/or Lys-77 (peptides 75–86) and Lys-148 (peptides 107–148 and 127–148). In contrast, acetylation of Lys-94 (peptide 91–106) was slightly enhanced. The apparently decreased labeling of peptide 1–86 and increased level of radioactivity associated with peptide 1–37 may simply reflect the fact that calmodulin acetylated in the presence of calcineurin, being less acetylated at lysines-13, -21, -30, -75, and -77, is more susceptible to clostripain cleavage at Arg-74.

Digestion with trypsin was needed to further define the relative extent of acetylation of individual peptides. The clostripain-resistant peptide 1–86 is cleaved by trypsin to a series of peptides corresponding to the amino-terminal fragment 1–37 and to peptide 75–86. Peptides eluting between 43 and 46 min correspond to peptide 1–37 acetylated either at Lys-13 and -30 or at all three lysyl residues and diacetylated peptide 1–30. A peak eluting between 42 and 43 min is peptide 1–30 acetylated at Lys-13. A peptide eluting at 29 min is acetylated fragment 14–30 (acetylated at Lys-21). Fragment 14–37 (either singly acetylated at Lys-30 or doubly at Lys-30 and -21) should elute at 32 and 34 min, respectively. These two peptides have not been obtained sufficiently pure to be identified definitely by amino acid composition. According to these assignments, the major radioactive peptides are monoacetylated, in agreement with the low level of acetylation.

The only lysine in peptide 1–37 whose reactivity is decreased with certainty by calcineurin is Lys-21 (Table I). The clostripain peptide 75–86 can be resistant to or cleaved by trypsin at Lys-75 or -77 depending on the state of acetylation of these two residues. A large peak eluting at 5 min, identified at Lys-Met-Lys, results from cleavage at Arg-74 and Lys-77 and is acetylated at Lys-75. It is labeled 4 times as much in the

Table II: Selection of Active Species of Acetylated Calmodulin by Calcineurin[a]

	acetylated calmodulin		bound:free selectivity ratio
	bound	free	
CaM sp act. (cpm/nmol)	760000	1170000	0.65
clostripain peptides (cpm)[b]			
1–37 (13, 21, 30)[c]	7100	9900	0.72
75–86a (75 or 77)	16000	24800	0.65
75–86b (75 + 77)	1000	1800	0.56
1–86[d] (13, 21, 30, 75, 77)	1100	3900	0.28
91–106 (94)	6300	7800	0.8
107–148 + 127–148 (148)	2200	3100	0.71
trypsin peptides (cpm)			
14–30 (21)	1900	3200	0.60
14–37 (21 or 30)	2600	3800	0.68
1–30 (13)	3200	4900	0.65
1–30, 1–37 (13, 21, 30)	550	1300	0.42
75–77 (75)	13900	21800	0.63
76–86 (77)	3700	5700	0.65
75–86 (75 + 77)	1000	1800	0.55
91–106 (94)	6500	8500	0.77
127–148 (148)	2400	3200	0.74

[a] Calcineurin was incubated with ^{3}H-acetylated calmodulin at a molar ratio of 1:1.6. Bound and free ^{3}H-acetylated calmodulins were isolated by glycerol gradient centrifugation and purified by reverse-phase HPLC. The distribution of acetyllysines in the bound and free fractions was compared by HPLC peptide mapping of clostripain and tryptic digests as described in Table I. [b] The cpm were corrected for recovery of radioactivity (68% and 87% for the clostripain and trypsin digests of bound calmodulin; 80% and 69% for the corresponding digests of free calmodulin) as described in the legend of Table I. [c] The numbers in parentheses indicate the acetylated lysines in the corresponding peptide.

absence of calcineurin as in its presence (Table I). The amount of fully acetylated peptide 75–86 is also greatly reduced by calcineurin. A small radioactive peak eluting at 16 min is probably peptide 76–86 labeled at Lys-77. The apparently increased labeling of this peptide in the presence of calcineurin can be explained by the lower level of acetylation of Lys-75, resulting in decreased production of the doubly modified peptide 75–86 and increased yield of acetylated peptide 76–86. Thus, as summarized in Table I, the reactivity of Lys-77 is unchanged in the presence of calcineurin, whereas that of Lys-75 is greatly diminished. The two peptides containing lysyl residues 94 and 148 exhibit elution profiles similar to those obtained with the corresponding clostripain peptides since Lys-94 is resistant to trypsin and Lys-148 is the COOH-terminal residue.

Effect of Limited Acetylation on the Ability of Calmodulin To Interact with Calcineurin. The reduced affinity of acetylated calmodulin for calcineurin could be due to the modification of specific lysines located at the interaction site(s) for calcineurin. The reduced reactivity of specific lysines in the presence of calcineurin is consistent with this model, but it could also result from a calcineurin-induced conformational change. To differentiate between these two models, we incubated calcineurin (1 μM) with 1.6 μM ^{3}H-acetylated calmodulin (0.35 mol of acetyllysine/mol) for 90 min at 0 °C. Calmodulin bound to calcineurin was separated from free calmodulin by glycerol gradient centrifugation, and the two calmodulin fractions (bound and free) were purified and quantitated by HPLC. The specific activity of bound calmodulin was 760 000 cpm/nmol, and that of the unbound calmodulin was 1 170 000 cpm/nmol (Table II). The lower specific activity of the bound calmodulin confirmed the reduced affinity of the more highly acetylated molecules for calcineurin. The distribution of acetylated lysines in the bound and free calmodulin was compared by peptide mapping (Table II). Since less than 3% of the molecules in the calmodulin preparation are acetylated at more than one position,[2] multiply acetylated species were almost undetectable in the peptide digests.

Analysis of tryptic and clostripain peptide maps demonstrated that the extent of acetylation of all lysyl residues was lower in the bound than in the unbound fraction. The extent of acetylation of Lys-75, Lys-77, and Lys-13, Lys-21, and Lys-30 in peptide 1–37 in the bound fraction was 63–64% of that in the free fraction. These results indicate that acetylation of any one of these lysyl residues reduced the binding of calmodulin to calcineurin, favoring recovery of these species in the free fraction. The level of acetylation of lysyl residue 148 in the bound fraction was 71–74% that of the free, suggesting less effect of this lysyl modification on interaction. Acetylation of Lys-94 exerted even less effect on binding, evidenced by only a 19–23% decreased level of acetylation in the bound fraction compared to the free. Because Lys-75 and -94 were the most extensively acetylated in the calmodulin preparation used for the experiment, the small decreased level of binding of calmodulin acetylated at Lys-94 may, in part, reflect the effect of the coexisting acetylation of Lys-75 to decrease binding of the molecules with two acetylated lysines.

Discussion

The reactivities of individual lysyl residues of calmodulin toward acetic anhydride are affected in different ways when calmodulin is complexed with calcineurin. Lys-77 reacts equally well in the complex as it does when the protein is free. The reactivities of lysine residues 21, 75, and 148 are markedly reduced in the complex whereas those of lysine-94 and, probably, lysine-13 and -30 are unchanged or slightly enhanced.

A similar approach has been used to identify the lysines involved in the interaction of calmodulin with β-endorphin and trifluoperazine (Giedroc et al., 1985). It was also found that the reactivities of Lys-75 and Lys-148 were greatly reduced in the presence of the interacting peptide or trifluoperazine and that the lysines in the amino-terminal half of the molecule were not affected. In the presence of trifluoperazine, acetylation of Lys-77 was also inhibited. On the basis of these experiments, Giedroc et al. postulated that Lys-75 and -148 are located in one of the interaction sites of calmodulin with β-endorphin and phenothiazine. Mastoporans which interact with calmodulin fragment 72–106 (Malencik et al., 1984) may also come in close contact with Lys-75. The similar effect of three different calmodulin targets, an enzyme, an inhibitory peptide, and the calmodulin antagonist, on the reactivity of two specific lysines, Lys-75 and -148, suggests that these lysines may be part of a common interacting domain. Early observations by Walsh and Stevens (1978), showing that carboxymethylation of Met-71, -72, and -76 abolishes the ability of calmodulin to stimulate cAMP phosphodiesterase, provided the first evidence of the importance of the central part of the calmodulin molecule (between the calcium binding domains II and III) for interaction with a target enzyme.

Thus, calmodulin may contain a common binding domain which encompasses the central helix and the C-terminus. On the other hand, a body of data exists which suggests that this hypothesis is too simple. Recent crystallographic data indicate that Lys-75 and Lys-148 are located on opposite sides of the central helix connecting the two calmodulin domains (Babu et al., 1985; W. Cook, personal communication). Lys-77, two-thirds of a helical turn away from Lys-75, is closer to Lys-148 than is Lys-75. Data obtained with half-size fragments of calmodulin (Newton et al., 1984) and with a covalent

adduct of norchlorpromazine and calmodulin (Newton et al., 1985) clearly show that different target proteins have different structural requirements for interaction with calmodulin. A fragment of calmodulin (77–124) lacks Lys-75, Met-76, and Lys-148 but interacts with phenothiazines (Head et al., 1982). Furthermore, the qualitatively different effects of several ligands on the reactivity of Lys-77 toward acetic anhydride, inhibition by phenothiazine, and no effect by β-endorphin or calcineurin, show that this lysyl residue does not interact with all targets similarly. Furthermore, evidence presented in the present study suggests that one of the lysyl residues located near the amino terminus of calmodulin, Lys-21, demonstrates reduced reactivity toward acetic anhydride in the calcineurin complex whereas it was unaffected in the complex with β-endorphin. Although one interpretation of these experiments is that the protected lysines are located in the interaction sites of calmodulin with its targets, an alternative possibility is that interaction with calcineurin or other targets induces a conformational change which affects lysine reactivity. Cooperativity between the drug binding sites has been reported by Mills et al. (1985). Klevit et al. (1985) recently showed that binding of a myosin light chain kinase fragment (M13) induces conformational changes in calmodulin.

To distinguish between interaction and conformational mechanisms, we prepared calmodulin acetylated at each of the lysyl residues, but with an average of less than 0.4 mol of modified lysine/mol. Under these conditions, calmodulin molecules were either unmodified (67%) or mostly modified at a single site (30% of calmodulin was monoacetylated and 3% diacetylated).[2] When challenged with this preparation of calmodulin, calcineurin preferentially bound unmodified calmodulin as reflected by the lower specific activity of bound compared to free calmodulin. This result would be expected if acetylation of some residues, located in the interaction sites, interferes with the ability of calmodulin to interact with calcineurin. However, when the acetylated lysines of bound and free calmodulins were identified by peptide mapping, almost no selection against particular lysyl residues was observed. Selection only against a population of calmodulin labeled at multiple sites, as a reason for this unselective discrimination, is unlikely since the acetylated calmodulin was essentially monoacetylated. Furthermore, the tryptic digests of the bound and free calmodulin did not reveal a significant difference in the number of multiply labeled peptides. Thus, acetylation of calmodulin results in a decreased affinity for calcineurin. Monoacetylation, at *any* lysine residue of calmodulin, produces some decrease in the tightness of binding to calcineurin. Isolated, monoacetylated, calmodulin was found to have 5–10-fold lower affinity than unmodified calmodulin for calcineurin. The smaller decrease in affinity observed with the monoacetylated species than with the fully acetylated one indicates that multiple modifications amplify the loss of affinity. Also, a larger selection ratio is consistently observed with peptides which are doubly modified such as peptide 75–86 or 14–37.

Thus, the protection of lysyl residues 21, 75, and 148 against acetylation by calcineurin may be due to calcineurin-induced structural change rather than to a direct contact of these lysines with calcineurin. As shown by Giedroc et al. (1985), Lys-94, Lys-148, and more particularly Lys-75 are sensitive probes for calcium-induced conformational transitions. According to the model of Dalgarno et al. (1984a), these residues are located in parts of the molecule (the ends of helix 4 and helix 8 and the β sheet formed between calcium binding loops III and IV) which undergo spatial rearangements when calmodulin binds calcium (Klevit et al., 1984). They are not among the residues located on the surfaces of the helices identified as parts of the phenothiazine binding sites (Dalgarno et al., 1984b). In the absence of calcium, all lysines of calmodulin are unreactive (Giedroc et al., 1985); their side chains may be buried in crevices along the surface of the protein while the charged amino groups are involved in salt bridges with neighboring carboxyl groups.

The reactivity of Lys-94 is increased dramatically and specifically at high ionic strength (in 50% sodium acetate). Other lysines, such as 75, and to a lesser extent 148, become reactive under more specific conditions such as calcium binding (Giedroc et al., 1985). Residues such as Lys-75 and -148, located in or close to the central helix of calmodulin, may act as sensors of calcium-induced structural transitions essential for interaction with target proteins. As proposed by Dalgarno et al. (1984a), the high degree of internal cooperativity predicts that binding of the proteins may generate conformational changes in the same part of the molecule. None of the modifications reported here are incompatible with activity; their effects can be overcome by increasing the concentration of target protein.

The effects of calcium and of any acetylation of the lysines on the structure and function of calmodulin suggest an important role for electrostatic interactions in these cooperative structural changes. Guanidination which does not modify the number of charged groups has a much smaller effect on the ability of calmodulin to interact with calcineurin and no effect on its ability to activate phosphodiesterase. It may not be surprising that acetylation, which decreases the number of positive charges, has an effect opposite to that of calcium, which increases the number of positive charges. Acetylation, which decreases the affinity of calmodulin for at least one enzyme, may counteract the effect of calcium. The internal cooperativity may help to explain why the modification of any lysine has a similar effect. These observations also emphasize the difficulties in dissecting overall structural changes common to all targets from those more subtle and specific which are associated with particular enzymes.

Added in Proof

After submission of the manuscript, Jackson et al. (1986) reported the protection of Lys-75, -21, and -148 of calmodulin against acetylation by myosin light kinase.

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Registry No. L-Lys, 56-87-1; Ca, 7440-70-2.

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