Modification of Calmodulin on Lys-75 by Carbamoylating Nitrosoureas*

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This paper describes characterization of the reaction of calmodulin with a series of nitrosoureas which are capable of releasing amine-reactive isocyanates of varying hydrophobic character. The site of calcium-dependent carbamoylation on calmodulin by the anti-neoplastic agent 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl CCNU) was determined to be Lys-75 as demonstrated using [ring-14C]methyl CCNU and sequence analysis of the sole labeled peptide obtained from tryptic digestion of reversed-phase high pressure liquid chromatography (HPLC)-purified radiolabeled calmodulin. CCNU, the 4-desmethylcyclohexyl derivative of methyl CCNU, and its reactive hydrolysis product, cyclohexyl isocyanate, were also determined to modify calmodulin in a similar manner and at the same site, as demonstrated by specific blockade of modification by the calmodulin antagonist calmidazolium. Nitrosoureas which release the less hydrophobic 4-hydroxy- and 4-carboxycyclohexyl isocyanates are unable to modify calmodulin at 25-fold higher concentrations than those required for modification with methyl CCNU, CCNU, or cyclohexyl isocyanate.

With this monomodified Lys-75 derivative, purified to homogeneity by HPLC, differential effects of modification on the activation of bovine brain 3',5'-cyclic nucleotide phosphodiesterase (phosphodiesterase) and human erythrocyte Ca**+-Mg**+-ATPase were observed. Compared to the amounts of native calmodulin needed, phosphodiesterase required 7-fold higher amounts of this derivative to reach maximal activation, whereas the activation of the ATPase was unaffected. Clearly, different regions of calmodulin are responsible for the activation of phosphodiesterase and the ATPase. We conclude that Lys-75 is not essential for the function of calmodulin but is in a region of the molecule involved in interaction with phosphodiesterase as well as the binding of certain hydrophobic calmodulin antagonists.

Although calmodulin is known to be involved in the calcium-dependent activation of many enzymes (for reviews see Refs. 1 and 2), there is only a limited understanding of the exact structures through which the binding to and activation of these enzymes are mediated. In the presence of micromolar calcium concentrations, calmodulin undergoes conformational changes associated with calcium binding that result in increased a-helicity and exposure of several hydrophobic sites (3). The recent determination of the three-dimensional structure of the calcium-replete protein as a highly asymmetric molecule with two calcium-binding structures at either end separated by a long central helix brings new insight into possible mechanisms of target enzyme activation (4).

The activation of calmodulin-regulated enzymes is inhibited by a wide variety of compounds including phenothiazines (6), naphthalene sulfonamides (6), anti-neoplastic agents (7), and hydrophobic peptides (8). The nitrosoureas, a class of chemotherapeutic agents that release alkylating and amine-directed carbamoylating reagents (for review, see Ref. 9), inhibit the ability of calmodulin to stimulate bovine brain 3',5'-cyclic nucleotide phosphodiesterase (phosphodiesterase) in a manner related to the carbamoylating activity of the nitrosoureas (10).

Differential trace labeling of calmodulin with acetic anhydride has demonstrated that Lys-75 undergoes a 25-fold increase in reactivity as the protein binds calcium (11). The reactive nature of Lys-75 and the propensity for carbamoylating nitrosoureas to act almost exclusively on lysyl residues in proteins (12) led us to evaluate and characterize the usefulness of these antineoplastic agents as affinity-directed calmodulin probes.

Other affinity-directed calmodulin probes have been synthesized by generating reactive phenothiazine derivatives. Norchlorpromazine isothiocyanate will modify calmodulin with a 1:1 stoichiometry in a calcium-dependent manner (13). The site of modification is believed to be Lys-75, as determined by amino acid analysis of tryptic peptides (14). The monooadduct, while incapable of activating phosphodiesterase, can act as a competitive inhibitor of native calmodulin for activation of the enzyme, much in the same manner as the amino-terminal half of calmodulin (15). The modification is blocked by trifluoperazine. Calmodulin has also been modified in a 1:1 stoichiometry with POS-TP, a reactive trifluoperazine derivative (16). The site of modification has been proposed to be Lys-148, as demonstrated by amino acid analysis of a labeled tryptic peptide (17). The calmodulin-mediated activation of phosphodiesterase and the ATPase are unaffected by modification at this site, whereas plant NAD kinase is not activated by this derivative (17).

Recently, the role of lysyl residues in the binding of calmodulin to two target enzymes has been studied by acetylation. Differential trace labeling showed substantial protection of Lys-75 from acetylation in the calmodulin-MLCK complex (18). Manalan and Klee (19) reported similar protection of this residue in the presence of calcineurin. However, Winkler and co-workers (20) have presented data suggesting that Lys-

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Calmodulin was isolated from bovine testes according to the procedure of Jamieson and Vanaman (21). Calmodulin-deficient 3',5'- cyclic nucleotide phosphodiesterase was partially purified from bovine brain according to the procedure of Klee and Krinks (22). Human erythrocyte Ca\(^{2+}\),Mg\(^{2+}\)-ATPase was isolated from outdated human blood, according to the procedure of Niggli and co-workers (23).

Nitrosoureas were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. [ring*\(^{14}C\)]Methyl CCNU. Through use of a series of carbamoylating nitrosoureas which release reactive isocyanates of differing hydrophobic character, we have further characterized the role of simple hydrophobic character of the modifying agent in directing calcium-dependent modification of calmodulin.

**EXPERIMENTAL PROCEDURES**

**Materials**

Calmodulin was isolated from bovine testes according to the procedure of Jamieson and Vanaman (21). Calmodulin-deficient 3',5'- cyclic nucleotide phosphodiesterase was partially purified from bovine brain according to the procedure of Klee and Krinks (22). Human erythrocyte Ca\(^{2+}\),Mg\(^{2+}\)-ATPase was isolated from outdated human blood, according to the procedure of Niggli and co-workers (23).

Nitrosoureas were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. [ring*\(^{14}C\)]Methyl CCNU (specific activity, 28 mCi/mmol) was obtained from Dr. Robert R. Engle of the Chemical Resources section of the National Cancer Institute. [ring*\(^{14}C\)]Methyl CCNU was followed by C-3 reversed-phase chromatography. Cyclohexyl isocyanate was purchased from Aldrich and dissolved in acetone prior to use. CCNU, 4-hydroxy-CCNU, and 4-carboxy-CCNU were obtained from the National Cancer Institute. Calmidazolium was purchased from Boehringer Mannheim, and t-l-chloro-3-(4-tosylamido)-4-phenyl-2-butanone-triphenyl and soybean trypsin inhibitor were purchased from Worthington. HPLC-grade acetonitrile was from Bur- dick and Jackson. Aqueous buffers, prepared with Milli-Q water, were filtered through 0.2-pm Millipore filters prior to use. HPLC columns (DEAE-TSK Spherogel 250 mm and Ultrapore RPSC C-3 250 mm) were purchased from Altex. Except as noted, all other chemicals and reagents were purchased from Sigma.

**Methods**

**Reaction of Nitrosoureas with Calmodulin**—The hydrophobic nitrosoureas CCNU and methyl CCNU (see Fig. 1 for structures and chemical names) were dissolved in absolute ethanol immediately prior to use and added to reaction mixtures containing 30 mM sodium borate (pH 8.0), 2 mM calcium chloride, and 1-10 \(\mu\)M bovine testes calmodulin. Incubation was performed for specified times at 37 °C. The less hydrophobic nitrosoureas 4-hydroxy CCNU and 4-carboxy CCNU (Fig. 1) were dissolved in 50% ethanol (in deionized water) before addition to the reaction mixtures. Cyclohexyl isocyanate was diluted with acetone and added to similar reaction mixtures. To assess the calcium dependence of the reactions, 2 \(\mu\)M EGTA was used to replace the 2 mM calcium chloride in each reaction mixture.

The time and methyl CCNU concentration dependence of the reaction of methyl CCNU with calmodulin was assessed by precipitating the calmodulin from a standard reaction mixture containing 10 \(\mu\)M calmodulin with 5% (v/v) trichloroacetic acid at various times.

1 The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea; HPLC, high pressure liquid chromatography; EGTA, \(\text{ethylenebis(oxyethylenenitrilo)tetraacetic acid; Pth, phenylthiohydantoin; POS-TP, 10-(3-propionylxosuccinimide)-2-(trifluoromethyl)phenothiazine; MLCK, myosin light chain kinase.}

Following initiation of the reaction with 10, 20, and 50 \(\mu\)M methyl CCNU. The precipitated protein was collected and dissolved in a minimum volume of 200 mM Tris-HCl (pH 8.0) and analyzed as described below. For reactions allowed to run to completion (2 h), extents of modification were quantified by direct analysis of the reaction mixture by HPLC following quenching of excess reagent by reaction with a 10-fold molar excess of lysine. The breakdown of CCNU and its 4-substituted derivatives was followed spectrophotometrically by the method of Forist (24), which measures the disappearance of the nitroso moiety.

HPLC analysis and purification of modified calmodulins—HPLC-based procedures were used not only to purify modified calmodulin but also as an analytical tool to follow the time course and extent of modification. For example, the modification of calmodulin with methyl CCNU was followed by C-3 reversed-phase chromatography. Following reaction of methyl CCNU and calmodulin in the pH 8.0 borate solution described above, native and modified calmodulins were separated on a C-3 reversed-phase column using a Varian 5000 liquid chromatograph. Separation of native and modified calmodulins was achieved using a 1%/min gradient increasing from 20 to 30% B with 10 mM dithiothreitol phosphate (pH 6.0), 2 mM EGTA, and 5% acetonitrile as buffer A, and 100% acetonitrile as the mobile phase.

Reversed-phase chromatography was unreliable for detecting and separating adducts formed with other less hydrophobic compounds (25). However, these derivatives were easily separated by anion-exchange HPLC as modification of lysine \(\varepsilon\)-amino groups leads to loss of a positive charge in the calmodulin molecule. Therefore, all nitrosoureas and cyclohexyl isocyanate were assayed for the ability to modify calmodulin using both reversed phase on C-3 and DEAE anion-exchange chromatography.

For anion-exchange chromatography, gradient elution from 40 to 60% B over 20 min was utilized to separate modified and unmodified calmodulins. Buffer A was 10 mM Tris-HCl (pH 7.0) and buffer B the same, with 0.8 M NaCl added.

Column effluents from both systems were monitored for UV-absorbing material using a Waters 441 UV detector at the wavelengths indicated in each trace. Peaks of UV-absorbing material were collected manually and analyzed as described in the following sections. Aliquots of peak fractions were counted by liquid scintillation spec-
trometry in the case of samples modified with radiolabeled compound agent. Relative amounts of native and modified calmodulin were determined by manual integration of peaks in the UV elution profiles or by automated peak integration using an HP 3932A integrator.

**Determination of Sites of Modification**—The site of modification was determined for the primary derivative resulting from the reaction of methyl CCNU with calmodulin. In a final concentration of 30 μM methyl CCNU, 1 μCi of [ring-14C]methyl CCNU was added to a standard reaction mixture containing 10 μM calmodulin. Following incubation, native and modified calmodulins were separated using reversed-phase HPLC as described above; the purified proteins were desalted by aminomethyl bis carbonate on Sephadex G-50 column, lyophilized, and the amount of peptide recovered quantified by amino acid analysis (26). The labeled peptide was then sequenced using an Applied Biosystems vapor-phase sequenator as described by Hunkapillar and co-workers (27). Aliquots from each cycle were assayed for radioactivity by liquid scintillation spectrometry. Pth-derivatives recovered from the sequenator were identified and quantified using a Du Pont Zorbax reversed-phase C-18 column by HPLC and automated peak integration (28).

**Functional Characterization of Modified Calmodulin**—Native and modified HPLC-purified calmodulins were assayed for the ability to activate phosphodiesterase by the procedure of Wallace et al. (29). In order to remove contaminating unmodified calmodulin, derivatives were repurified on a new C-3 reversed-phase column as described above. For use in these assays, protein calmodulin concentrations were determined by amino acid analysis (26). The resulting protein was desalted by gel filtration and concentrated by lyophilization prior to assay. The ATPase was assayed for stimulation by calmodulin and its derivative following reconstitution into phospholipid vesicles using a coupled enzyme determination of released inorganic phosphate according to the procedure of Niggli and co-workers (23). Standard curves for control levels of activation were obtained using HPLC-purified native calmodulin.

**Studies with Calmidazolium**—The ability of calmidazolium, a calcium-dependent calmodulin antagonist (30), to block the modifications with methyl CCNU, cyclohexyl isocyanate, and CCNU was assayed by adding calmidazolium dissolved in a minimum volume of absolute ethanol (<5% of total volume) to the standard reaction mixture containing 1 μM calmodulin and 20 μM carbamoylating agent. Extent of modification was determined by automatic integration of native and modified calmodulin peaks eluted from the C-3 column using a Hewlett-Packard 1090 liquid chromatograph and integrator with effluents monitored at 230 nm. Parallel assays of dose-dependent calmidazolium inhibition of phosphodiesterase activation by calmodulin were performed by adding the compound dissolved in absolute ethanol (<5% of total volume) to the standard phosphodiesterase assay and using amounts of calmodulin sufficient to provide submaximal levels of activation.

**RESULTS**

**Calmodulin Modifications**—The reaction of calmodulin with methyl CCNU has been characterized in detail. Fig. 2 shows reversed-phase HPLC analysis of reaction mixtures containing 10 μM calmodulin and 20 μM methyl CCNU incubated for 2 h at pH 8.0 in the presence or absence of Ca²⁺. A single major new peak (Peak 2), eluting from C-3 at 27% acetonitrile, was formed in the presence of 2 mM Ca²⁺ (lower chromatogram). Only material eluting at 25% acetonitrile, the position of unmodified calmodulin (Peak 1), was observed following incubation in 2 mM EGTA (upper chromatogram). Studies performed with [14C]-labeled methyl CCNU described below confirmed that Peak 1 was unmodified calmodulin and demonstrated that Peak 2 contained only a single specific calmodulin derivative which will be referred to as "monoaduct" hereafter. The elution of this monoaduct from C-3 at higher acetonitrile concentration than the unmodified protein is indicative of the incorporation of a hydrophobic moiety into the protein.

The rate of modification is dependent on the methyl CCNU concentration employed in the reaction mixture (Fig. 3). Nearly total modification of 10 μM calmodulin was obtained with 50 μM methyl CCNU at the 120-min time point with formation of the monoadduct almost exclusively. The modification is also strongly time-dependent (Fig. 3). Measurement of exact rates of modification is difficult with these reagents because of the fact that the amine-reactive isocyanates must first be generated by hydrolysis of the parent compound and they are subsequently destroyed by reaction with hydroxyl ion (Fig. 1).

The calcium-dependent modification of calmodulin by CCNU, cyclohexyl isocyanate, 4-hydroxy CCNU, and 4-carboxy CCNU was assayed by HPLC analysis of reaction mixtures. Cyclohexyl isocyanate and CCNU modify calmodulin in a completely calcium-dependent manner, indistinguishable from that observed for methyl CCNU modification. The less hydrophobic nitrosoureas were unable to generate any adduct formation at any concentration or pH tested. These results are summarized in Table I. These compounds were tested for modification by both reversed-phase C-3 and DEAE anion-exchange HPLC. Breakdown of all nitrosoureas was followed spectrophotometrically, and good agreement with published values for CCNU decomposition (24) was obtained for all nitrosoureas.

**Determination of Site of Modification**—In order to fully characterize the modification of calmodulin by methyl CCNU, 50 nmol of the bovine testes protein was reacted with 30 μM methyl CCNU containing 1 μCi of the [ring-14C]compound (final specific activity, 11,826 cpm/nmol) in a 5.0-ml reaction mixture, as described under "Methods." Separation of reaction products by reversed-phase HPLC on Altec C-3 (Fig. 4A) yielded four peaks of UV-absorbing material, three of which also contained radioactivity. The first peak (peak 1) contained no radiolabel and eluted at the position of unmodified calmodulin (25% acetonitrile).

As before, the major peak of UV-absorbing material (Peak 2) eluted at 27% acetonitrile which was also coincident with
eluting at higher acetonitrile concentrations in the profile. Two smaller peaks of radioactive material (peaks 3 and 4) were also obtained. CH isocyanate refers to cyclohexyl isocyanate.

Reactions were started by addition of methyl CCNU and 5% (v/v) trichloroacetic acid. The percentage of modified calmodulin was determined by weighing and totaling the various peaks representing unmodified or modified calmodulin or by automated peak integration. Reactions were started by addition of methyl CCNU in absolute ethanol.

The time and methyl CCNU concentration dependence of the modification of 10 μM calmodulin. Reactions were stopped at the indicated times by precipitation of reaction mixtures with 5% (v/v) trichloroacetic acid. The percentage of modified calmodulin was determined by weighing and totaling the various peaks representing unmodified or modified calmodulin or by automated peak integration. Reactions were started by addition of methyl CCNU in absolute ethanol.

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The concentration of compound which when completely reacted gives 50% modification of 10 μM calmodulin. The concentration of compound which when completely reacted gives 50% modification of 10 μM calmodulin.

**TABLE I**

Properties of calmodulin reaction with selected aminesor directed reagents

| Compound          | Conc. for 50% modification | HPLC separation | Modification calcium dependent |
|-------------------|-----------------------------|-----------------|--------------------------------|
|                   | μM                          | %               | %                             |
| Methyl CCNU       | 20                          | RP-C-3          | 95                             |
|                  |                              |                 | 100                            |
| CCNU              | 20                          | RP-C-3          | 95                             |
|                  |                              |                 | 100                            |
| 4-Hydroxy CCNU    | 500                         | DEAE            | —*                            |
|                  |                              |                 | NM*                            |
| 4-Carboxy CCNU    | 500                         | DEAE            | —*                            |
|                  |                              |                 | NM*                            |
| CH isocyanate     | 10                          | RP-C-3          | 95                             |
|                  |                              |                 | 100                            |

*For all experiments, a calmodulin concentration of 10 μM was used. CH isocyanate refers to cyclohexyl isocyanate.

The concentration of compound which when completely reacted gives 50% modification of 10 μM calmodulin.

—* means not done.

NM refers to no modification detected.

a large peak of 14C radiolabel. The specific radioactivity of the modified calmodulin present in this peak was determined by amino acid composition analysis and scintillation counting to be 11,400 cpm/nmol, within experimental error of that expected for incorporation of 1 mol of reagent/mol of protein. Two smaller peaks of radioactive material (peaks 3 and 4), eluting at higher acetonitrile concentrations in the profile shown in Fig. 4A, contained considerably smaller amounts of calmodulin as judged both by the corresponding peaks of UV-absorbing material and by amino acid analysis. Based on their specific radioactivities estimated from the latter (~19,000 cpm/nmol), the calmodulin in these peaks was modified by 2 or more mol of reagent/mol of protein. A total of 60% of the calmodulin applied to the separation shown in Fig. 4A was recovered in peaks 1-4, similar to recoveries routinely obtained for the unmodified protein from Altex C-3 columns.

The major [14C]methyl CCNU-labeled calmodulin derivative, isolated as peak 2 from multiple separations identical to that in Fig. 4A, was further characterized to demonstrate that it was specifically modified. Fig. 4B shows the elution profile obtained for a trypsin digest of this radiolabeled peak 2 material separated by reversed-phase HPLC. A single major radioactive peptide was obtained eluting from C-3 at 33 min into gradient elution performed under conditions where the intact protein eluted at 46 min. Greater than 90% of the applied radioactivity was recovered in the fraction containing this peak. The amino acid composition of this material was in excellent agreement, except for the presence of a nucleoside, with that expected for the peptide spanning residues Lys-75 through Arg-86 of bovine calmodulin, assuming that the detection of only 1 mol of lysine/mol of peptide resulted from incomplete modification of one of the two lysyl residues in this sequence (not shown).

In order to unequivocally identify the site of modification, a sample of this peptide was subjected to automated Edman degradation on an Applied Biosystems Vapor-Phase Sequencer. The 14C radiolabel and Pth-derivative content obtained at each cycle were determined and are shown in Table II. Approximately 80% of the radiolabel recovered was obtained in the fraction from the first cycle where a Pth-lysine having a different elution time than unmodified Pth-lysine was recovered. All the radioactivity recovered in this cycle was accounted for in this altered Pth-lysine peak. Cycle 2 contained the remainder of the radiolabel as well as Pth-methionine which was shown to be devoid of 14C by scintillation counting of the Pth-methionine peak collected from this separation. The remainder of the cycles contained no radiolabel and gave those Pth-derivatives on HPLC analysis expected for the sequence Lys-75 through Arg-86.

It should be noted that the modified Pth-lysine present in the first cycle was not identified with the Du Pont Zorbax C-18 column used initially to resolve Pth-derivatives. An identical labeled peptide was isolated from a subsequent trypsin digest of [14C]methyl CCNU-labeled calmodulin and the Pth-derivatives obtained from vapor-phase sequencing resolved isocratically on a Du Pont Bioseries Pth column (31). A peak...
### Table II

| Cycle no. | Sequence of [ring-14C]methyl CCNU-labeled tryptic peptide |
|-----------|----------------------------------------------------------|
| 1         | Lys-Met-Lys-Asp-Thr-Asp-Thr-Glu-Glu-Glu-Ile-Glu-Arg     |
| 2         | Ly-Lys-Asp-Thr-Asp-Thr-Glu-Glu-Glu-Ile-Glu-Arg         |
| 3         | Lys-Asp-Thr-Asp-Thr-Glu-Glu-Glu-Ile-Glu-Arg          |
| 4         | Asp-Thr-Asp-Thr-Glu-Glu-Glu-Ile-Glu-Arg            |
| 5         | Thr-Asp-Thr-Glu-Glu-Ile-Glu-Arg                      |
| 6         | Thr-Glu-Glu-Ile-Glu-Arg                              |
| 7         | Glu-Glu-Ile-Glu-Arg                                  |
| 8         | Glu-Ile-Glu-Arg                                     |
| 9         | Glu-Ile-Arg                                        |
| 10        | Glu-Arg                                            |
| 11        | Arg                                                |
| 12        | Glu, Arg, and Glu                                    |
| 13        | Glu, Arg, and Glu                                    |

| nmol of Pth-derivative detected | cpm/cycle |
|----------------------------------|-----------|
| ND                               | 1284      |
| 0.09                             | 410       |
| 0.04                             | ND        |
| 0.19                             | ND        |
| 0.03                             | 0.13      |
| 0.10                             | ND        |
| 0.05                             | 0.12      |

a Taken from the known sequence of mammalian calmodulin (46).
b nmol of each Pth-derivative recovered/cycle, as outlined under “Experimental Procedures.” ND means none detected.

For each cycle, an aliquot was assayed for radioactivity by liquid scintillation spectrometry. The numbers listed refer to counts/min above background.

**Fig. 5.** Calmidazolium inhibition of the modification of 1 μM calmodulin by 20 μM methyl CCNU (circles) and 20 μM cyclohexyl isocyanate (triangles). The effect of calmidazolium on the activation of phosphodiesterase by 36 ng of calmodulin (squares). On the abscissa, % of control refers to the amount of inhibition of modification observed for complete reaction at each of the calmidazolium concentrations shown on the ordinate. The numbers for each data point represent the average of n = 3 ± S.E. for methyl CCNU and the average of two determinations for cyclohexyl isocyanate modification. Extents of modification were determined by automated peak integration for modified and unmodified calmodulins using reversed-phase HPLC.

**Fig. 6.** A, the activation of phosphodiesterase by native HPLC-purified calmodulin (closed circles) and purified Lys-75-modified calmodulin (open squares) for native calmodulin (closed circles) and by Lys-75-modified calmodulin (open squares). Each point represents the average of three determinations.

**DISCUSSION**

The formation of specific calmodulin derivatives wherein the site(s) of modification have been unambiguously determined continues to be important in relating calmodulin structures to both functional and chemical properties. Through use of [ring-14C]methyl CCNU, the calcium-dependent modification of calmodulin by methyl CCNU was almost completely inhibited by addition of 1 μM calmidazolium to the reaction mixture. The extent of modification produced following 2 h of incubation when various calmodulins at each of the calmidazolium concentrations were added is shown in Fig. 5. A similar curve was observed for the calcium-dependent modification of 1 μM calmodulin by 20 μM cyclohexyl isocyanate (closed triangles), with the same concentration required for 50% inhibition in both curves. Calcium-dependent CCNU modification was inhibited over an identical range of calmidazolium concentrations (not shown). The effect of a range of calmidazolium concentrations on the ability of 36 ng of calmodulin to activate phosphodiesterase is shown by the closed squares. Again, 1 μM calmidazolium was sufficient to inhibit the majority of the activation.

**Effects of Modification on Function** — To determine the effect of the Lys-75 modification on the functional integrity of calmodulin, calmodulin derivatives were repurified on a new C-3 column and used in phosphodiesterase and ATPase activation assays. Derivatives prepared in this way contained no unmodified calmodulin as determined by HPLC. The effect of UV-absorbing material, eluting earlier than Pth-lysine and containing >90% of the radioactivity present, was found in the first cycle (data not shown).

**Effect of Calmidazolium on Modifications** — The calcium-dependent modification of calmodulin by 20 μM methyl CCNU was almost completely inhibited by addition of 1 μM calmidazolium to the reaction mixture. The extent of modification produced following 2 h of incubation when various concentrations of calmidazolium were added is shown in Fig. 5. A similar curve was observed for the calcium-dependent modification of 1 μM calmodulin by 20 μM cyclohexyl isocyanate (closed triangles), with the same concentration required for 50% inhibition in both curves. Calcium-dependent CCNU modification was inhibited over an identical range of calmidazolium concentrations (not shown). The effect of a range of calmidazolium concentrations on the ability of 36 ng of calmodulin to activate phosphodiesterase is shown by the closed squares. Again, 1 μM calmidazolium was sufficient to inhibit the majority of the activation.

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The effect of calmidazolium on the activation of phosphodiesterase by 36 ng of calmodulin (squares). On the abscissa, % of control refers to the amount of inhibition of modification observed for complete reaction at each of the calmidazolium concentrations shown on the ordinate. The numbers for each data point represent the average of n = 3 ± S.E. for methyl CCNU and the average of two determinations for cyclohexyl isocyanate modification. Extents of modification were determined by automated peak integration for modified and unmodified calmodulins using reversed-phase HPLC.

The effect of calmidazolium on the activation of phosphodiesterase by native HPLC-purified calmodulin (closed circles) and purified Lys-75-modified calmodulin (closed triangles). Phosphodiesterase activity refers to nmol of cAMP hydrolyzed/min/mg of phosphodiesterase. Each point represents the average of three determinations.

**Fig. 6.** A, the activation of phosphodiesterase by native HPLC-purified calmodulin (closed circles) and purified Lys-75-modified calmodulin (closed triangles). Phosphodiesterase activity refers to nmol of cAMP hydrolyzed/min/mg of phosphodiesterase. Each point represents the average of three determinations.

**DISCUSSION**

The formation of specific calmodulin derivatives wherein the site(s) of modification have been unambiguously determined continues to be important in relating calmodulin structures to both functional and chemical properties. Through use of [ring-14C]methyl CCNU, the calcium-dependent modifica-
tion of calmodulin has been shown to occur on Lys-75. We have been able to trace the modification from incorporation of the label into the intact protein to a single labeled peptide and ultimately to a modified Pth-lysine derivative which behaves chromatographically different than unmodified Pht-lysine. Hydrolysates of other proteins (albumin, histones) treated with CCNU have yielded cyclohexylcarbamoyllysine as a modification product (32). It therefore is likely that 4-methylocyclohexylcarbamoyllysine is the structure of the Lys-75 monoadduct on calmodulin obtained through reaction with methyl CCNU. The altered retention time of the resulting Pth-4-methylocyclohexylcarbamoyllysine (25) is also anticipated due to the prevention of incorporation of an additional Pth moiety on the ε-amino group.

Both CCNU and cyclohexyl isocyanate, the carbamoylating moiety of CCNU, are capable of specific calcium-dependent modification of calmodulin. Modification by the above compounds and methyl CCNU is inhibited to the same extent by identical concentrations of the calmodulin antagonist calmidazolium, indicating that modification for all of these compounds most likely occurs at Lys-75. Additionally, these calmodulin derivatives all activate phosphodiesterase equally.

That the modification occur at Lys-75 is not particularly surprising, since this residue has been shown to undergo a 25-fold increase in reactivity toward acetic anhydride when calmodulin binds calcium (11). Hydrophobic reactive phenothiazine derivatives such as norchlorpromazine isothiocyanate (13) and a fluorenyl-based spin labeling reagent (33) are also likely to modify calmodulin on Lys-75, although both also modify a second lysyl residue (probably Lys-148) at a substantial rate. It is somewhat surprising that another reactive phenothiazine derivative, POS-TP, produces calcium-dependent modification of calmodulin preferentially on Lys-148 (17). The selectivity of this reagent for Lys-148 may reflect the orientation of the reactive moiety of the POS-TP in that the reagent may still bind to the hydrophobic pocket near Lys-75, but the reactive moiety is inaccessible to the ε-amino group of this residue.

Interestingly, Lys-77, located in an environment expected to be similar to that occupied by Lys-75, is one of the least reactive residues on calmodulin (11). We have as yet been unable to demonstrate any modification of this residue. Its unreactivity may be due to the formation of a salt bridge with Asp-80. These structures are anticipated to participate in the stabilization of the central helix (34). The hydrophobicity of the modifying agent may be important in directing modification at Lys-75 in that the side chain of this residue appears to be oriented toward the proposed hydrophobic pocket formed by α-helices II and II in the three-dimensional structure of the calmodulin molecule (4). This may explain the failure of the less hydrophobic nitrilotriacetic acid and 4-hydroxy and 4-carboxy CCNU derivatives, to modify calmodulin at Lys-75. The failure of these compounds to produce any modification is not understood at this time. That calmodulin concentrations equal to the calmodulin concentration employed (1 μM) were able to almost completely inhibit modification at Lys-75 may indicate that the hydrophobic modifying agents such as methyl CCNU must first be able to bind to calmodulin prior to reaction.

Since calmidazolium blocks the specific modification of calmodulin at Lys-75, as well as calmodulin-stimulated phosphodiesterase activation over similar concentration ranges, it seems likely that this region of the molecule plays important roles in mediating both the binding of standard hydrophobic calmodulin inhibitors to the hydrophobic site located in the NH2-terminal half of the molecule and the activation of phosphodiesterase. Modification at Lys-148 by POS-TP is also inhibited by trifluoperazine (17). Previous studies have shown that each half of the calmodulin molecule contains 1 phenothiazine binding site (35). Studies with N-hydroxysuccinimidothiobenzoate have shown that calcium-dependent modification at Lys-94 is not blocked by calmidazolium.

The modification of Lys-75 by methyl CCNU results in a derivative that is still able to stimulate phosphodiesterase maximally, but much greater amounts are required. Since the charge on this ε-amino group is destroyed by this reaction, it is clearly not essential for maximal stimulation of phosphodiesterase. Due to the effect observed, however, Lys-75 and/or the structures adjacent to it are involved in this interaction. LARGER aromatic calmodulin derivatives with substituents on Lys-75 (13), such as norchlorpromazine isothiocyanate, are unable to activate phosphodiesterase at all. Based on the three-dimensional structure of calmodulin, Lys-75 is anticipated to reside at the beginning of helix IV. The side chain of this residue has been observed to point directly into the hydrophobic "pocket" formed by helices II and III (4). It is attractive to speculate that the charge on this residue is important in orienting the formation of the phosphodiesterase-calmodulin complex. The final interaction, which dictates the "tightness" of the binding, would be expected to involve both the central helix and the hydrophobic pocket. The central helix (IV) may function by conferring α-helical structure on the calmodulin-binding region of phosphodiesterase, as has been observed for a synthetic peptide representing the calmodulin-binding domain of myosin light chain kinase (37).

The hydrophobic pocket may serve as an anchor by binding aromatic residues such as tryptophan which have been observed in calmodulin binding domains of several dependent enzymes (e.g. MLCK (38) and muscle phosphofructokinase (39)) as well as small peptide antagonists (e.g. mellitin (40) and mastoparan (41, 42)).

The size and possibly the aromaticity of the adduct on Lys-75 may be crucial determinants in the ability of these derivatives to stimulate phosphodiesterase. These effects may be related to the ability of these derivatives to substitute for binding of target enzymes to the hydrophobic pocket. By reaction of calmodulin with a phenothiazine-based spin-labeling reagent Jackson and Puett (33) have obtained a Lys-75 and -148 dimodified calmodulin. That treatment of this calmodulin with sodium hydroxide, which removes the phenothiazine portion of this adduct, restores some of the ability of this calmodulin to activate phosphodiesterase supports the above contention. We also have observed this phenomenon when an HPLC-purified calmodulin adduct, believed to be modified at Lys-75 by reaction of calmodulin with a cleavable heterobifunctional arylazo compound, is treated with hydroxylamine to remove the arylazo portion of the derivative (25). Therefore, the resulting smaller adducts, while still lacking a positive charge on Lys-75, can have a free hydrophobic pocket, which enables maximal activation.

Acetylation by acetic anhydride to a stoichiometry of 6.6 mol of [14C]acetyllysine/mol of calmodulin inhibits the ability of calmodulin to stimulate phosphodiesterase 7-fold (18). This extent of inhibition is similar to that observed with methyl CCNU-modified calmodulin. Both derivatives were able to stimulate the enzyme maximally if sufficient amounts were employed. The fact that studies of acetylated derivatives were performed with unpurified mixtures of products presumably acetylated to varying extents precludes precise interpretation. Therefore, while modification of Lys-75 with reagents that destroy the charge on the ε-amino group may be expected to

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reduce the affinity of calmodulin for phosphodiesterase, they do not prevent calmodulin from activating the enzyme maximally.

A model based on a computer-predicted structure of calmodulin has postulated electrostatic interactions and sequencing of hydrophobic residues to be the primary driving forces of calmodulin-basic amphiphilic peptide interactions (43). As discussed above, the data discussed herein are not inconsistent with this model. The modification of charge-containing lysyl residues, while reducing the apparent affinity for interaction, does not prevent maximal stimulation from occurring if large amounts of calmodulin are employed, thus indicating that the electrostatic binding mediated by these residues, while not of primary importance in the two types of calmodulin-target enzyme interactions described, still plays some role. As the derivative is fully able to activate the ATPase, it is unlikely that this modification perturbs the overall structure of calmodulin.

The calmodulin-ATPase interaction is clearly mediated by different structures on calmodulin than the phosphodiesterase-calmodulin interaction. The modification of Lys 75 did not affect the activation of the ATPase at all, thus indicating that this region is not involved in the activation of this enzyme. On the basis of the limited ability of trypsin-generated fragments of calmodulin encompassing residues 78–148 and 1–106 to stimulate the ATPase (44), calcium-binding domain III would seem to be of primary importance, although the necessary structures within this domain remain unclear. The ATPase clearly binds to different structures within calmodulin than enzymes such as phosphodiesterase, whose activation by calmodulin is strongly inhibited by synthetic MLCK calmodulin-binding peptides (37). In this regard, we have recently identified what appears to be an inhibitory action, does not prevent maximal stimulation from occurring of calmodulin-basic amphiphilic peptide interactions (43). As the necessary structures within this domain remain unclear, 262, 15466–15471

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