The Effects of Calcium Site Occupancy and Reagent Length on Reactivity of Calmodulin Lysyl Residues with Heterobifunctional Aryl Azides

MAPPING INTERACTION DOMAINS WITH SPECIFIC CALMODULIN PHOTOPROBE DERIVATIVES*

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The relationship of structural and functional moieties on calmodulin is important in all venues of cell activity. In this study, we investigate the effect of lysine modification on calmodulin function. Azidosalicylic reagents containing different "linker-arms" lengths, between the photoactive terminus and an amine-reactive N-hydroxysuccinimidyl ester moiety were used to modify calmodulin lysines at three different positions in a calcium-dependent manner. The short cross-linker, (ASNE-2 (where ASNE represents azidosalicylate N-hydroxysuccinimidyl ester), modifies Lys-75, whereas the longer reagent, ASNE-6, modifies lysines 21, 75, and 94. The modification of these different lysines is shown to be calcium-dependent. At 1–100 μM levels of calcium, only Lys-94 is modified, suggesting that modification of this residue is directed by both the binding of calcium to calcium-binding loops III and IV and the hydrophobic pocket exposed between these two loops as a result of calcium binding. At higher calcium concentrations (>200 μM), where sites I and II become filled, modification of Lys-21 or Lys-75 also was observed. All the modified calmodulins were able to stimulate 3',5'-cyclic-nucleotide phosphodiesterase fully although the K onset for the Lys-75 and Lys-21 derivatives increased 10- and 50-fold, respectively. None of the modifications affected the activation of erythrocyte plasma membrane Ca2+-ATPase.

The ASNE-2 Lys-75 derivative as well as the ASNE-6 Lys-21 and Lys-94 derivatives did not show efficient calcium-dependent photocross-linking to this enzyme.

Although calmodulin (CaM) is known to be involved in the calcium-dependent activation of many enzymes and cellular processes (Klee and Vanaman, 1982), the mechanism of activation of target enzymes is still being elucidated. The three-dimensional x-ray crystal structure of the calcium-replete molecule indicates that CaM exists as a rigid protein with two globular lobes, each containing two calcium-binding sites, at either end of a long central helix (Babu et al., 1985, 1988). However, substantial data now exist suggesting that this rigid structure does not represent the active conformation of Ca2+/CaM in solution. Persechini and Kretsinger (1988a) demonstrated that the two halves of a recombinant CaM that contain cysteinyl residues inserted at positions 3 and 146, 37 Å apart in the crystal structure, could be cross-linked with the thiol-directed reagent bismaleimidohexane, which could span at most 19 Å. Furthermore, this cross-linked derivative retained the ability to activate rabbit skeletal muscle myosin light chain kinase. O’Neil et al. (1989) demonstrated that a synthetic peptide corresponding to the calmodulin-binding domain of myosin light chain kinase but containing photoreactive p-benzoylphenylalanine derivatives at either end would photolabel methionyl residues in the NH2- and COOH-terminal hydrophobic pockets of CaM, an observation that further supported the folded conformation of Ca++/CaM in solution. Persechini and Kretsinger (1988b) have proposed a model for this interaction with myosin light chain kinase based on extensive molecular modeling and use of the folded CaM structure that suggests that flexibility in the central helix of CaM, particularly in the backbone folding in the region around residue 80, allows for CaM to bind to and activate its target enzymes.

Amino acid substitution, deletion, and insertion with genetically engineered CaMs as well as chemical modification studies have provided substantial evidence that various target enzymes interact with different regions of the CaM molecule (Newton et al., 1985; Mann and Vanaman, 1988, 1989; George et al., 1990; Kink et al., 1990; VanBerkum et al., 1990; Persechini et al., 1991). For example, modification of Lys-75 with cyclohexyl isocyanate produced a 7-fold increase in the concentration of CaM required for 50% activation of bovine brain 3',5'-cyclic-nucleotide phosphodiesterase (Mann and Vanaman, 1988), whereas activation of the erythrocyte Ca2+-ATPase was not affected. Conversely, Lys-94 biotinylation increased the CaM concentration required for Ca2+-ATPase activation 4-fold, whereas 3',5'-cyclic-nucleotide phosphodiesterase activation was unaffected (Mann and Vanaman, 1989).

Lysine 75 modification and the functional consequences are particularly interesting because of the presence of its side chain at the mouth of a hydrophobic "pocket" composed of both aliphatic side chains and a well-ordered set of phenylalanine side chains. Previous studies indicated that hydrophobic interaction is required for modification at Lys-75 (Mann and Vanaman, 1988). NMR studies of the binding of pheno-
thiazines to CaM indicated that the drugs may intercalate into the stacked aromatic side chains that line each pocket (Anderson et al., 1983; Dalgarno et al., 1984). Interestingly, differential effects on the degree of 3',5'-cyclic-nucleotide phosphodiesterase activation have been demonstrated for aliphatic eraser aromatic adducts of Lys-75. The norchlorpromazine Lys-75 derivative of CaM is unable to activate 3',5'-cyclic-nucleotide phosphodiesterase (Newton et al., 1983), whereas the cyclohexyl adduct still gives maximal activation, but shows decreased binding affinity (Mann and Vanaman, 1988).

Despite the numerous studies to date, the structural and steric considerations that direct the modification of specific lysyl residues in CaM and the relationship of Ca2+ site occupancy to lysine reactivity remain to be defined. This study, which shows decreased binding affinity (Mann and Vanaman, 1988), whereas the cyclohexyl adduct still gives maximal activation, may be important in dictating lysyl reactivity, which can be accounted for based on the known three-dimensional crystal structure of CaM.

The resulting photoactive CaM derivatives are used to further examine the interaction of CaM with two target enzymes, 3',5'-cyclic-nucleotide phosphodiesterase and erythrocyte membrane Ca2+-ATPase. Photocross-linking studies with the latter enzyme also demonstrate that the length and site of attachment of the photoprobe as well as its photochemical properties are important in determining the efficiency with which such CaM derivatives cross-link to target proteins.

EXPERIMENTAL PROCEDURES

Materials

Calmodulin was isolated from bovine tissues according to the procedure of Jamieson and Vanaman (1979). Activator-deficient bovine brain 3',5'-cyclic-nucleotide phosphodiesterase was purchased from Sigma. Erythrocyte plasma membrane Ca2+-ATPase was isolated from fresh pig blood according to the method of Niggli et al. (1987). 1-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Burdick & Jackson Laboratories Inc. Aqueous buffers were prepared with Milli-Q water and filtered through 0.22-μm Millicron filters before use. Reverse-phase C18 columns and C4 bulk resin were purchased from Waters. C18 columns (4 × 250 mm) were packed at 5000 p.s.i. in 100% Fisher HPLC-grade methanol using a Shandon HPLC column packer. Mono Q columns were purchased from Pharmacia LKB Biotechnology Inc. The ASNE reagents (shown in Fig. 1) were synthesized and characterized as described by Imai et al. (1990). Enzyme mobeads were purchased from Bio-Rad. Carrier-free Na2125I (17 Ci/mg iodine) was purchased from ICN. All other chemicals were purchased from Sigma.

Methods

Reaction of ASNE Compounds with CaM—Immediately before use, the ASNE reagents were dissolved in acetonitrile at a concentration of 1 mg/ml. For most studies, 2 (ASNE-6) or 5 (ASNE-2) molar eq of reagent were added to reaction mixtures containing 30 mM HEPES (pH 7.4), 2 mM CaCl2 (or 2 mM EGTA), and 10 μM CaM. The conditions were altered to prepare large quantities of ASNE-6 monoadducts: those mixtures contained 50 μM ASNE-6 (5 molar eq) plus 1.38 mM EGTA and 1.36 mM CaCl2 to obtain a free Ca2+ concentration of 2.1 × 10⁻⁶ M (based on studies of the Ca2+ dependence of modification of specific lysyl residues described under "Results"). Reactions were allowed to proceed at 25 °C for 2 h, at which time they were quenched by the addition of lysine to a final concentration of 10 mM. The extent of reaction was monitored using reverse-phase HPLC as described by Mann and Vanaman (1988), which separates unmodified CaM from CaM derivatives because of the addition of the hydrophobic aryl azide group. The ASNE reagents exhibit a UV absorbance maximum at 310 nm due to the aryl azide, and the ratios of 310:230 nm absorbances reflect the extent of modification of CaM derivatives.

Assessment of Calcium Dependence of Modification of Specific Lysyl Residues by ASNE-6 Reagent—Reactions were performed in 50 mM HEPES (pH 7.4) and 2 mM EGTA with increasing CaCl2 concentrations, fixed using CaCl2/EGTA buffers as described by Goldstein (1979), in a total volume of 250 μl. The entire reaction was subjected to reverse-phase C18 HPLC. The native CaM and adduct peaks were integrated, and the percent of total CaM present in each peak was calculated based on the sum of the areas of the native, monoadduct, and polyadduct peaks detected at 320 nm.

Purification of Monoadducts—CaM-ASNE monoadducts were isolated on a large scale using a combination of fast protein liquid chromatography and HPLC techniques (see Miniprint). Large-scale reaction mixtures containing 6 mg of CaM were resolved on an anion-exchange Mono Q fast protein liquid chromatography column. Further analysis by reverse-phase C18 (ASNE-2) and C4 (ASNE-6) HPLC showed that the monoadducts of the ASNE-2 reaction and the low calcium ASNE-6 reaction could be purified by application to the Mono Q column. One of the monoadducts from the high calcium ASNE-6 reaction was pure after Mono Q chromatography. The other monoadduct required further purification on C4 HPLC with multiple runs. These purified monoadducts were desalted and characterized.

Determination of Modification Sites—Purified CaM-ASNE monoadducts were subjected to trypsin digestion as described by Vanaman (1983). Modified CaM (100-500 μg) in 100 mM (pH 8.0) was digested with 1:20 trypsin/CaM for 2 h (twice) at 37 °C. The resulting mixture of peptides was applied to a phenyl μBondapak reverse-phase HPLC column for peptide isolation (Vanaman, 1983). The purified peptide was separated at 25 °C using a 1% linear gradient of Buffer A (10 mM NaH2PO4, pH 6.5, 2 mM EGTA) and Buffer B (5 mM NaH2PO4, pH 6.5, 1 mM EGTA, 50% acetonitrile) from 0 to 50% Buffer B. Modified peptides were identified on the basis of UV absorbance at 310 nm. Fractions containing the modified peptides were desalted over the same column by elution with a gradient (1%/min) formed with 0.1% (v/v) trifluoroacetic acid and increasing amounts (0-50%) of 88% (v/v) acetonitrile, 0.05% (v/v) trifluoroacetic acid. In each case, a single peak absorbing at both 230 and 310 nm was obtained. This peptide-containing fraction was subjected to automated sequence analysis on an Applied Biosystems Model 477A Protein Sequencer at the University of Kentucky Macromolecular Structure Analysis Facility.

Functional Characterization of CaM-ASNE Monoadducts—CaM-ASNE monoadducts were tested for their ability to activate bovine brain 3',5'-cyclic-nucleotide phosphodiesterase and the porcine erythrocyte plasma membrane Ca2+-ATPase. The 3',5'-cyclic-nucleotide phosphodiesterase assays were performed as described by Wallace et al. (1983), except that cAMP hydrolysis was measured by release of free phosphate (Lanzetta et al., 1979) from 5'-AMP. Ca2+-ATPase

![Fig. 1. Chemical structure of ASNE reagents. The n refers to the number of methylene groups in the linker arm region of the reagent (i.e. succinimimidyl N-[2-[4-azidosalicyloyloxy]ethyl]suberate (n = 6) is represented by the abbreviation ASNE-6).](http://www.jbc.org/)

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assays were done according to the procedure of Niggli et al. (1987), with released phosphate also detected using Lanzetta reagent (Lanzetta et al., 1979). Concentrations of stock solutions of CaM and CaM-ASNE monoadducts were determined by amino acid analysis following acid hydrolysis as previously described (Vanaman, 1985).

Cross-linking CaM-ASNE Monoadducts to ATPase—CaM-ASNE monoadducts were radiiodinated by the Enzymobead method according to the procedure supplied by Bio-Rad. One hundred μCi of 125I (17 Ci/mg iodine) were used for each iodination. Iodinated monoadducts were desalted before use. Specific activities of each of these monoadducts were determined to be as follows: Lys-21 CaM, 3.1 mCi/μmol; Lys-75 CaM, 1.65 mCi/μmol; and Lys-94 CaM, 1.2 mCi/μmol. Cross-linking was performed as described by Imai et al. (1990), except that purified monoadducts were used. Each reaction mixture contained 1.5 μM CaM-ATPase, 6 μM radiolabeled CaM-ASNE monoadduct, 30 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl2, 0.05% (v/v) Triton X-100, 5% (v/v) glycerol, and 0.5 mg/ml phosphatidylincholine. Cross-linking reactions performed in the presence of calcium contained 100 μM CaCl2. Reactions without calcium contained 10 mM EGTA. Native CaM competition reactions contained 12 μM (2-fold molar excess) unmodified CaM. Photolysis was done using a hand-held ultraviolet light (4000 milliwatts/cm², Mineralite model UVS-40). Cross-linking reactions were resolved on 7.5% SDS-polyacrylamide gel (Laemmli, 1970). The gel was dried, autoradiographed for 16 h, rehydrated, and stained with silver (Morrissey, 1981).

RESULTS

Modification of CaM by ASNE Reagents—Fig. 2 shows reverse-phase C₄ HPLC analysis of the products obtained when CaM was reacted with ASNE-2 and ASNE-6 in the presence (upper panels) or absence (lower panels) of calcium. Reactions were carried out at pH 7.4 for 2 h as described under “Methods.” The solid line shows 230 nm absorbance, and the dashed line shows 310 nm absorbance. The asterisk in each trace indicates the elution position of unmodified CaM, determined from a prior analysis and detected as a peak at 230 nm with little 310 nm absorbance. Products eluting prior to 10 min were shown to be hydrolyzed ASNE or adducts of ASNE with free lysine used to quench excess reagent (data not shown). The products eluting later than unmodified CaM contained 1.5 μM CaM and had both 230 and 310 nm absorbance were CaM-ASNE adducts. The major products (indicated by arrows in upper panels) were determined to be monoadducts by virtue of the ratio of the UV absorbance at 310 versus 230 nm and by subsequent sequence analysis (see below). The other products eluting after the monoadducts were determined to be polyadducts on the basis of their 310:230 nm absorbance ratios and peptide analysis on purified derivatives (data not shown).

In the absence of calcium (Fig. 2, lower panels), <5% of the calmodulin was modified by either reagent. As shown in Fig. 2 (upper left panel), reaction of CaM with ASNE-2 (5 molar eq) in the presence of 2 mM CaCl2 yielded one major new peak of material on HPLC analysis. Further characterization (see below) showed this peak to contain a single CaM monoadduct modified at Lys-75. Reaction with ASNE-6 (2 molar eq) under similar conditions yielded two major peaks on HPLC analysis (Fig. 2, upper right panel). Further separation on C₄ columns and subsequent analyses showed that three distinct monoadducts of CaM were present in these two peaks. Fig. 3 shows a typical separation of CaM and the three CaM-ASNE-6 monoadducts using reverse-phase C₄ HPLC. Separation of these monoadducts was determined to be optimal at 18 °C (data not shown), the temperature used in the separation shown here. The peaks eluting at 13.1 min (indicated by arrow) showed little 310 nm absorbance and was unmodified CaM. The peaks eluting at 14.2, 14.6, and 15.2 min, respectively, were shown to be unique CaM-ASNE-6 monoadducts modified at Lys-21, Lys-75, or Lys-94 as discussed below. For ease of discussion, these monoadducts will be referred to as 1, 2, or 3 based on their order of elution from C₄ columns. The relative abundance of these three monoadducts was dependent on the concentration of calcium present during the reaction as discussed below.

Calcium Dependence of Modification by ASNE-6—Previous analysis of the reaction of CaM and ASNE-6 showed the production of three major monoadducts (see Fig. 3). The generation of these monoadducts was shown to be dependent on the calcium concentration in the reaction mixture as illustrated in Fig. 4. CaM modification reactions were performed as described before, except that the calcium concentration was varied over a range of 10⁻⁴ to 10⁻² M by the use of a calcium/EGTA buffering system (Goldstein, 1979). The reactions were performed in triplicate and were analyzed by C₄ HPLC at 18 °C. Previous analysis had determined the elution position of unmodified CaM as well as each of the monoadducts and diadducts. Using the peak areas obtained
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FIG. 3. Separation of CaM and three CaM-ASNE-6 monoadducts using reverse-phase C4 HPLC. Separation was achieved using the same buffers and conditions as in the C3 separations (see Fig. 2), except that the column was cooled to 18°C. As before, the left axis (-) indicates the UV absorbance scale at 230 nm, whereas the right axis (---) indicates the UV absorbance scale at 310 nm. The arrow indicates the elution position of unmodified CaM, whereas the numbers above the peaks denote the three CaM-ASNE-6 monoadducts.

FIG. 4. Effect of calcium concentration on specificity of modification of CaM by ASNE-6. Values were determined using the peak rates from C4 analysis of reactions. O, amount of CaM that remained unmodified in the reaction; O, Lys-21 (peak 1), Lys-75 (peak 3), and Lys-94 (peak 2) modifications, respectively. Free calcium concentration is depicted by pCa (-log of the free calcium). Points were determined as the average of the analysis of three separate reactions. Error bars show standard deviation.

by integration, the amount of total CaM recovered was calculated (values were within 10% in each of three separate analyses). The individual peak areas were used to determine the fraction of total CaM that each monoadduct comprised. These values are plotted as a function of pCa in Fig. 4. The disappearance of native CaM is indicated (O). At a pCa value of 8.2, monoadduct 2 began to appear (■). This was the only monoadduct formed until the pCa reached 4.2, where monoadducts 1 and 3 began to appear ( and 0, respectively). Diadducts also were formed efficiently beginning at a pCa of 4.2, which accounts for the parallel decline in the amount of both monoadduct 2 and unmodified CaM at the higher Ca2+ concentrations.

Determination of Sites of Modification—Using the calcium concentrations indicated in the previous experiment, large-scale preparations of each CaM-ASNE-6 monoadduct as well as of the CaM-ASNE-2 monoadduct were prepared as described in the Miniprint. The pure monoadducts were subjected to trypsin digestion, and the resulting peptides were purified by phenyl μBondapak HPLC monitored at 230 and 310 nm. The resulting peptide maps are shown in Fig. 5. For ease of presentation, only the 310 nm traces are shown for the modified CaMs (Fig. 5, C–F). The 230 and 310 nm traces obtained with the trypsin digest of the unmodified protein are shown in Fig. 5 (A and B, respectively). The 230 nm traces obtained with digests of the monoadducts looked similar to Fig. 5A, except that a new peak having both 230 and 310 nm absorbance was present in each case. Unmodified CaM peptides showed very little 310 nm absorbance (Fig. 5B). Fig. 5 (C–E) shows the HPLC analyses of the trypsin digests of the CaM-ASNE monoadducts using detection at 310 nm. The ASNE-2 monoadduct digest is shown in Fig. 5C, where one major 310 nm absorbing peak is seen eluting at 37.2 min. The ASNE-6 monoadducts are shown in Fig. 5 (D–F). One major 310 nm absorbing peak was seen for each monoadduct analyzed.

The fraction from each separation containing the 310 nm

FIG. 5. Phenyl μBondapak HPLC separation of trypsin digests of native CaM, CaM-ASNE-2 monoadduct, and CaM-ASNE-6 monoadducts. Separations were achieved using a 1%/min gradient of Buffer B (5 mM NaH2PO4, (pH 6.5), 1 mM EGTA, 50% acetonitrile) and Buffer A (10 mM NaH2PO4 (pH 6.5), 2 mM EGTA). The absorbance scale of each separation is indicated on the left axis of each panel. A and B show the 230 and 310 nm absorbance traces, respectively, of the trypsin peptide map of native CaM. B–F, 310 nm traces of digests of CaM-ASNE derivatives. C shows that of the CaM-ASNE-2 monoadduct. D–F show the maps of ASNE-6 monoadducts 1–3, respectively. Arrows indicate the modified peptide peaks.
absorbing material was pooled and reapplied to the phenyl
µBondapak column to remove salt. Only one peak was seen
for each fraction with the acidic buffer system used for elution
during this desalting step (data not shown). The peak fraction
was collected and submitted to sequence analysis as described
under “Methods.” The sequences were identified as to their
position in the known CaM sequence (Watterson et al., 1980)
as shown in Table I. In each case, a cycle corresponding to a
lysyl residue showed no identifiable phenylthiohydantoin-
derivative on HPLC analysis, but did produce an unidentified
peak. In the case of the ASNE 2 monoadduct, this peak
occurred at 19.5 min, whereas in the sequence of the ASNE-
6-containing peptides, the peak appeared at ~27 min (see
Miniprint). The CaM-ASNE-2 monoadduct is modified at
Lys-75, as would be expected from previous modification
studies (Mann and Vanaman, 1988). CaM-ASNE-6 monoadd-
uct 3 is also modified at Lys-75. CaM-ASNE-6 monoadduct
1 is modified at Lys-21, whereas the modification that takes
place at saturating calcium concentrations occurs at Lys-
94, yielding monoadduct 2.

**Functional Studies Using CaM-ASNE Monoadducts**—Since
the modifications were in different regions of the CaM mole-
cule, functional studies with these adducts were undertaken
to determine whether modification altered binding to and
activation of two enzymes that appear to interact with differ-
ent regions of the CaM molecule, bovine brain 3',5'-'cyclic-
nucleotide phosphodiesterase and porcine erythrocyte mem-
brane Ca2+-ATPase. Figs. 6 and 7 show the results of assays
using native CaM and the characterized CaM-ASNE mono-
adducts. The CaM-ASNE monoadducts activated the Ca2+-
ATPase in a manner indistinguishable from native CaM (see
An 8-10-fold increase in the concentration of both the CaM-
and Lys-21 affected the ability of CaM to activate the enzyme.

Modification at Lys-94 (+) required to stimulate 3',5'-cyclic-nucleotide phosphodiesterase activation.

**Cross-linking Studies with CaM-ASNE Monoadducts**

**TABLE I**

| Sequence of ASNE-2-labeled adduct | Cycle No. | nmol PTH detected |
|-----------------------------------|-----------|-------------------|
| Expected sequence (75–77)α | 1 2 3 | Lys-Met-Lys |
| Sequence of ASNE-6-labeled adduct 3 | Cycle No. | nmol PTH detected |
| Expected sequence (75–77)α | 1 2 3 | Lys-Met-Lys |
| Sequence of ASNE-6-labeled adduct 2 | Cycle No. | nmol PTH detected |
| Expected sequence (91–106) | 1 2 3 4 5 6 7 8 9 10  | Val-Phe-Asp-Lys-Asp-Gly-Asn-Gly-Tyr-Ile-Ser-Ala-Ala-Glu-Leu-Arg |
| | .37 .49 .20 ND | .15 .12 .06 .08 .11 .06 .10 .08 .07 .02 .03 ND |
| Sequence of ASNE-6-labeled adduct 1 | Cycle No. | nmol PTH detected |
| Expected sequence (14–30) | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 | Glu-Ala-Phe-Ser-Leu-Phe-Asp-Lys-Asp-Gly-Asp-Thr-Ile-Thr-Thr-Lys |
| | .03 1.0 .94 .34 1.3 3.80 .05 ND | .05 .19 .04 .14 .10 .44 .06 .04 .04 |

α Taken from the known sequence of mammalian calmodulin (Watterson et al., 1980).

The nanomoles of phenylthiohydantoin (PTH)-derivative present in each cycle were quantified using an Applied Biosystems Model 477 Automated Protein Sequencer.

ND, none detected.
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Total ATPase activation is defined as the full activity shown with unmodified CaM. Each point is the average of at least three separate assays. Error bars represent standard deviation.

Fig. 6. Activation of porcine Ca\(^{2+}/\)Mg\(^{2+}\)-ATPase by native CaM and three CaM-ASNE-6 monoadducts. O, activation by unmodified CaM; ●, CaM-ASNE-6 Lys-21 monoadduct; ○, CaM-ASNE-6 Lys-75 monoadduct; •, CaM-ASNE-6 Lys-94 monoadduct. Total ATPase activation is the total activity seen with saturating native CaM. Each point is the average of at least three separate assays. Error bars represent standard deviation.

Fig. 7. Activation of 3',5'-cyclic-nucleotide phosphodiesterase with CaM-ASNE monoadducts. O, activation of 3',5'-cyclic-nucleotide phosphodiesterase (PDE) by unmodified CaM; ●, CaM-ASNE-6 Lys-94 monoadduct; ○, CaM-ASNE-2 Lys-75 monoadduct; •, CaM-ASNE-6 Lys-21 monoadduct. Total enzyme activation is the total activity seen with saturating native CaM.

Previous studies indicated that hydrophobic and/or aromatic compounds interact with the hydrophobic pockets in the amino- and carboxyl-terminal globular domains of calmodulin (LaPorte et al., 1980; Weiss et al., 1982; Newton et al., 1986; Mann and Vanaman, 1989). The Ca\(^{2+}\)-dependent modification of these 3 residues by ASNE-6 prompted further study into the factors directing lysine modification in CaM.

Previous studies indicated that hydrophobic and/or aromatic compounds interact with the hydrophobic pockets in the amino- and carboxyl-terminal globular domains of calmodulin (LaPorte et al., 1980; Weiss et al., 1982; Newton et al., 1986; Mann and Vanaman, 1989). To determine if the ASNE reagents were also interacting with the hydrophobic pockets, competition studies were performed with the calmodulin antagonist calmidazolium. One eq of calmidazolium was shown to inhibit the reaction of CaM with 5 molar eq of ASNE-2 (Mann, 1987). The reaction of ASNE-6 and CaM was also inhibited by calmidazolium, but the concentration of calmidazolium required to completely inhibit the reaction exceeded the solubility of calmidazolium in an aqueous solution (data not shown). In addition, this calmidazolium competition appeared to be biphasic, indicative of two binding events. The amino-terminal modifications were abolished at calmidazolium concentrations comparable to those seen with ASNE-2. The Lys-94 modification was not affected until higher calmidazolium concentrations were reached, which suggested that the modification of these 3 lysines was controlled by two separate phenomena.

The calcium dependence of the reaction was determined to investigate further the reactions of ASNE-6 with CaM. At subsaturating calcium concentrations, only the CaM-ASNE-6 Lys-94 monoadduct was formed. At saturating calcium concentrations, Lys-21 and Lys-75 were also modified. This calcium dependence provides further evidence that the carboxyl- and amino-terminal pairs of Ca\(^{2+}\)-binding sites have high and low Ca\(^{2+}\) affinities, respectively, as determined by direct ligand binding (Watterson et al., 1976; Crouch and Klee, 1980; Klee, 1988), NMR (Klevit, 1983; Linse et al., 1991), and specific Ca\(^{2+}\)-site mutagenesis (Beckingham, 1991). These results also provide definitive evidence for separate conformational changes accompanying Ca\(^{2+}\) binding in the
Ca2+- and amino-terminal globular domains, resulting in the sequential exposure of each respective hydrophobic pocket.

As part of this study, we examined the diadducts formed in the reaction of ASNE-6 with CaM that were favored in the presence of increased ASNE concentrations. Preliminary examination of diadducts formed in the reactions of CaM with ASNE-6 in the presence first of low, then high calcium concentrations yielded derivatives modified either at Lys-94 and Lys-21 or at Lys-94 and Lys-75 (data not shown). No diadduct modified at both Lys-75 and Lys-21 was detected, indicating that the reaction of the reagents with CaM was specific for each hydrophobic pocket. Information on reaction rates at each of these lysyls has yet to be determined at any calcium concentration, but 2.5-fold less (on a molar basis) ASNE-6 reagent is required to modify 50% of the CaM compared with ASNE-2 in the presence of saturating calcium. Whether this is due to the availability of multiple modification sites for ASNE-6 or an increased binding affinity of the reagent has yet to be determined.

Molecular modeling was employed to address further the specificity of lysyl modification. In the fully staggered conformation, the ASNE-2 adduct measures 16.1 Å from the Ca of lysine to the terminal azide nitrogen, whereas that with ASNE-6 measures 21 Å. This difference in length appears to be the key factor that determines which CaM lysyl residues are modified by the two reagents. Fig. 9 shows the 2.2 Å crystal structure of calmodulin (Babu et al., 1988). Lys-21 resides at the “top” of calcium-binding loop I in Fig. 9. Lys-75 sits at the “base” of the amino-terminal hydrophobic pocket. Lys-94 in calcium-binding loop III is in an analogous position to Lys-21 in calcium-binding loop I.

The distances between the lysyl residues and the hydrophobic pockets were measured using the solvent surface depiction of the crystal structure of calmodulin. As shown in Fig. 10 (upper panel), the amino-terminal hydrophobic pocket is a crescent-shaped cleft defined by the stacked aromatic rings of the phenylalanine residues. Measurements made from the ε-amino group of Lys-21 over the lip of the hydrophobic pocket and into its center was ~18 Å. This would allow the ASNE-6 (but not the ASNE-2) reagent to reach from the pocket to the Lys-21 ε-amino group. Lys-75, on the other hand, is positioned at the base of the pocket and is accessible to all the reagents.

The carboxyl-terminal hydrophobic pocket (Fig. 10, lower panel) is cup-shaped and lined with the aromatic rings of the tyrosyl and phenylalanine residues. Measurements indicated that the distance from Lys-94 -NH2 to the center of the aromatic cluster is ~18-19 Å. Once again, only the ASNE-6 reagent is long enough to span the distance from this hydrophobic pocket to Lys-94. The importance of these distance relationships is highlighted by the fact that the ASNE-4 reagent, which is only 2.5 Å shorter than ASNE-6, modifies primarily Lys-75 in a Ca2+-dependent manner (Mann, 1987).

The availability of photoactive derivatives of CaM modified at specific sites in the molecule with reagents of different lengths provides substantial opportunity for studying CaM-target protein interaction. The results of photocross-linking studies with erythrocyte membrane Ca2+-ATPase presented in Fig. 8 show that the ASNE-6 Lys-75 monoadduct is capable of efficient (40%) cross-linking, whereas the corresponding CaM-ASNE-2 derivative gives little cross-linking despite the fact that both Lys-75 monoadducts activate Ca2+-ATPase activity with indistinguishable dose-response curves. The actual cross-linking efficiency with CaM-ASNE-6 actually may be higher than 40% as the Ca2+-ATPase used in the cross-linking reaction is present in micelles, presumably in two orientations, with the CaM-binding region either exposed to the buffer or on the inside of the micelle.

The ASNE-6 Lys-21 and Lys-94 adducts gave very little Ca2+-dependent cross-linking to the Ca2+-ATPase. In preliminary studies (Imai et al., 1990), a mixture of the CaM-ASNE-6 monoadducts were used in cross-linking experiments, yielding cross-linking efficiency of only 8% (Imai et al., 1990). The results presented in the present work clearly demonstrate the need to work with purified and characterized modified derivatives if the cross-linking efficiencies are to be assessed.

The nature of the reactive species generated on photolysis of the aryl azide is also extremely important in governing cross-linking efficiencies between donor and target structures. Previous studies (Crocker et al., 1990) using the CaM Lys-75 monoadduct of succinimidyl 2-(4-azido-2,3,5,6-tetrafluorophenyl)thiazole-4-carboxylate demonstrated that this perfluorinated aryl azide derivative photocross-linked to the membrane Ca2+-ATPase with an efficiency approaching that of the ASNE-6 Lys-75 derivative despite the fact that it is the shortest of the reagents thus far employed (9.8 Å from lysine Ca to the azido group). Whereas conformational and steric considerations cannot be excluded, one logical explanation for these apparently disparate results is the nature of the intermediates formed on photolysis of these reagents.

The nitrene generated on photolysis of aryl azides has been shown to generate a ring-expanded dehydroazepine as the putative cross-linking species (Torres et al., 1986; Leyva et al., 1986; Shields et al., 1987; Schuster and Liang, 1987). The
dehydroazepine is an electrophilic alkylation reagent that does not have the ability to insert into C-H and C-C bonds as does a highly reactive singlet nitrone. The perfluorinated aryl azide was selected specifically to avoid ring expansion and thereby provide access to a reactive singlet nitrone (Crocker et al., 1990). It is interesting to speculate that the shorter monoadduct may not be able to bind calcium as effectively as the diadduct would be expected to span a maximum distance of 27 Å based on computer modeling. The amino acid sequence of the region of the Ca²⁺-ATPase labeled on photolysis of the complex with this modified CaM derivative contained a number of potential sites for cross-linking using CaM modified with the Denny-Jaffe reagent (Jaffe et al., 1980). The lysine adduct of this reagent is more reactive to proteolysis than is either unmodified CaM or the apoCaM or Ca⁺⁺/CaM. Preliminary experiments showed that addition of 10 mM calcium improves the ability of CaM-Lys-21 to activate the 3',5'-cyclic-nucleotide phosphodiesterase (data not shown).

These studies illustrate, once again, that CaM may interact with different target enzymes in different ways. The model of

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Calcium Site Occupancy Dictates Calmodulin Lysine Reactivity

**INTRODUCTION**

The modification reactions described in the text were carried out to allow for the isolation of milligram quantities of monomodified calmodulin for characterization and cross-linking studies. Modifications were performed on 5 mg of native calmodulin in 30 ml reaction mixtures. The reactions were carried out in a MES buffer at pH 6.5, which is the optimal pH for the enzymatic reaction. The reaction mixtures were incubated at 37°C for 60 min. The reaction was terminated by the addition of an isopropanol to a final concentration of 10%, followed by application to Mono Q resin. The products were separated by gel filtration on a Superose 6 column. The fractions containing the mono-lysyl-modified calmodulin were collected and subjected to further purification by high performance liquid chromatography (HPLC) and mass spectrometry. The molar ratio of the modified to the unmodified calmodulin was determined by mass spectrometry. The mass spectrometry data were analyzed using a Waters Micromass tzQ Quadrupole Linear Ion Trap mass spectrometer. The mass spectrometry data were analyzed using the m/z values of the modified and unmodified calmodulin peaks. The ratio of the modified to the unmodified calmodulin was determined by dividing the m/z value of the modified peak by the m/z value of the unmodified peak. The ratio was then multiplied by 100 to obtain the percentage of modified calmodulin. The percentage of modified calmodulin was then used to calculate the number of lysines modified in the reaction. The number of lysines modified was calculated by dividing the m/z value of the modified peak by the m/z value of the unmodified peak and multiplying by the total number of lysines in calmodulin. The m/z value of the modified peak was obtained by subtracting the m/z value of the unmodified peak from the m/z value of the modified peak. The m/z value of the unmodified peak was obtained by subtracting the m/z value of the modified peak from the m/z value of the unmodified peak. The number of lysines modified was then calculated by multiplying the ratio of the modified to the unmodified calmodulin by the total number of lysines in calmodulin.
Calcium Site Occupancy Dictates Calmodulin Lysine Reactivity

Figure 14. C-3 analysis of MCHO-Q fraction from the renaturation of ASME-2/CM
monomoduct. This analysis was performed as described in Figure 12. The elution position of unmodified 
CM (previously determined) is indicated by the arrow. The elution position of the 
monomoduct is indicated by the asterisk.

Figure 15. MCHO-Q analysis of the ASME-2/CM reaction at saturating calcium 
concentration. The MCHO-Q separation was performed as described in Figure 11. The fractions 
collected are indicated by the numbers above the peaks and are bounded by the braces.

Figure 16. C-4 analysis of the ASME-2/CM reaction products from MCHO-Q. The C-4 analyses 
were performed as previously described for C-3 separation (Figure 17). Separation was 
achieved using a 15-mm gradient of Buffer B (100 mM acetate) and Buffer A (50 mM 
phosphate, 25 mM sodium phosphate, 0.01% SDS). The elution position of native 
CM is indicated by the arrow. The elution position of the monomoduct is indicated by 
the asterisk. Eluent was monitored at 220 nm (solid lines) and 310 nm (dashed lines). 
The left axis represents the 220 nm absorbance scale while the right axis depicts the 310 nm 
absorbance scale. Upper panel: Fraction 1, Figure 15. Middle panel: Fraction 2, Figure 15. 
Lower panel: Fraction 3, Figure 15.

Figure 17. MCHO-Q analysis of ASME-2/CM reaction at saturating calcium concentration. 
The MCHO-Q separation was achieved as described in Figure 15. The gradient was 
shortened to 5-50% Buffer B. The 4 fractions collected are indicated by the numbers above the peaks. 
The glass panels indicate the boundaries of each Fraction.

Figure 18. C-4 analysis of ASME-2/CM reaction products separated by MCHO-Q. The analyses 
were performed as described in Figure 16. The MCHO-Q fraction numbers are indicated in the 
upper left corner of each trace. Eluent was monitored at 220 nm (solid lines) and 310 nm 
(dashed lines). The elution position of native CM is indicated by the arrow. Elution 
positions of the monomoducts are indicated by the asterisks. The left and right axes show 
the 220 nm and 310 nm absorbance scales, respectively.

Figure 19. C-4 analysis of fractions containing ASME-2/CM monomoducts #1 and #7. Analyses 
were performed as described in Figure 16. Eluent was monitored at 220 nm (solid lines) and 310 nm 
(dashed lines). Panel A shows the analysis of the fraction containing ASME-2/CM 
monomoduct #1 while Panel B shows that containing ASME-2/CM Monomoduct #7. The arrows 
denote the monomoduct peaks.

Figure 20. On-line FAB-mass analysis of ASME-2 modified tryptic peptide Lys-Met-Lys. 
The traces shown were obtained on sequence analyses of the 310 nm absorbance tryptic peptide 
isolated from ASME-2 modified CM as shown in Figure 5 (right panel). The analysis 
was performed on an Applied Biosystems, Inc. Model 470A Protein Sequencer equipped with an on-line 
HPLC. Separation of FAB-mass sites was performed as described by Applied Biosystems, Inc. 

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L D Dwyer, P J Crocker, D S Watt and T C Vanaman

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