Preparation, characterization and biological properties of biotinylated derivatives of calmodulin

Joseph W. POLLI* and Melvin L. BILLINGSLEY†

Department of Pharmacology and Center for Cell and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

Biotinylated derivatives of calmodulin (CaM) were prepared and their biological properties characterized by using enzyme assays, affinity and hydrophobic-interaction chromatography. Several *N*-hydroxysuccinimidobiotin derivatives [sulphosuccinimidobiotin (sulpho-NHS) and sulphosuccinimido-6-(biotinamido)hexanoate (BNHS-LC)] differing in spacer arm length were used to modify CaM. The shorter-spacer-arm CaM derivative (sulpho-CaM) activated CaM-dependent cyclic nucleotide phosphodiesterase and CaM-dependent protein kinase II; preincubation with avidin blocked its ability to activate these enzymes. The extended-spacer-arm derivative (BNHS-LC-CaM) activated CaM-dependent enzymes both in the presence and in the absence of avidin, suggesting that the longer spacer arm diminished steric effects from avidin preincubation. Other biotinylated CaM derivatives were prepared with biotinylated tyrosine and/or histidine residues (diazobenzoylbioctin; DBB-CaM) or nucleophilic sites (photobiotin acetate; photo-CaM). These derivatives activated CaM-dependent enzymes in the presence and in the absence of avidin. Oriented affinity columns were constructed with covalently immobilized avidin complexed to each biotinylated CaM derivative. The chromatographic profiles obtained revealed that each column interacted with a specific subset of CaM-binding proteins. Elution profiles of biotinyl CaM derivatives on phenyl-Sepharose hydrophobic-interaction chromatography suggested that several derivatives displayed diminished binding to the matrix in the presence of Ca^{2+} . Development and characterization of a series of biotinylated CaM molecules can be used to identify domains of CaM that interact with specific CaM-dependent enzymes.

INTRODUCTION

Calmodulin (CaM) is a 148-residue 17 kDa acidic Ca^{2+} -binding protein (Cheung, 1982; Manalan & Klee, 1984; Stoclet *et al.*, 1987). Structurally, CaM is a single polypeptide containing four Ca^{2+} -binding sites, which lie within two globular end regions connected by a long extended α -helical backbone (Babu *et al.*, 1985; Kretsinger *et al.*, 1986). CaM is a member of the EF-hand family of Ca^{2+} -binding proteins (Persechini *et al.*, 1989), and each globular region contains a pair of EF-hand structures (helix–loop–helix motif). Each EF-hand contains a Ca^{2+} -binding domain. A unique structural feature of the CaM molecule is the solvent-accessible central helix. This central helix has an important functional role in the activation of CaM-dependent proteins. Initial studies suggested that the central helix is the region of CaM that binds to and activates CaM-binding proteins (CaM-BPs) (Babu *et al.*, 1985; Kretsinger *et al.*, 1986). However, this model of binding and activation of CaM-BPs does not explain several experimental observations. First, CaM contains two hydrophobic domains, but is only found in a 1:1 association with CaM-BPs (Blumenthal *et al.*, 1985; Klevit *et al.*, 1985). Secondly, CaM is able to bind to and activate a variety of proteins that have little amino acid sequence similarity in their respective CaM-binding domains (Manalan & Klee, 1984; Stoclet *et al.*, 1987; Persechini & Kretsinger, 1988).

A second model has been proposed for the binding of CaM to its target proteins that can account for these observations (O'Neil & DeGrado, 1990). The model employs the central helix as a

flexible tether. In the presence of Ca^{2+} , the central helix bends bring the hydrophobic domain of each globular lobe together to form a single hydrophobic patch. Further, when the putative CaM-binding domains of individual CaM-BPs are configured to form α -helices, a Basic Amphiphilic Alpha-helical motif ('BAA' motif) emerges for the majority of CaM-BPs. Clustered to one side of the helix are basic positively charged amino acid residues and hydrophobic amino acid residues are clustered on the opposite side. This suggests that the CaM–CaM-BP interaction utilizes hydrophobic and electrostatic forces (O'Neil & DeGrado, 1985). The hydrophobic pocket formed by the two hydrophobic domains in CaM would then interact with the α -helical structure of the CaM-binding domain in CaM-BPs. Specificity of binding for individual CaM-BPs could occur by altering the degree of bending of the central helix and the spatial orientation of amino acid side chains located within the hydrophobic pocket of CaM. Therefore CaM can specifically bind to and activate a wide variety of cellular proteins with a 1:1 stoichiometry without the requirement of a consensus CaM-binding sequence common to all CaM-BPs.

Even though a considerable amount is known about the sequence, structure and nature of the Ca^{2+} -induced conformational change in CaM, the specific domains involved in CaM–protein interactions remain to be fully determined. Chemical modification of specific residues of CaM has been used to identify specific domains and amino acid residues of CaM that are involved in binding to various CaM-BPs. Specific modifications include acylation (Giedroc *et al.*, 1985; Winkler *et al.*,

Abbreviations used: CaM, calmodulin; CaM-BP, calmodulin-binding protein; CaM-PDE, CaM-dependent 3',5'-(cyclic)nucleotide phosphodiesterase; CaM-kinase II, CaM-dependent protein kinase II; BNHS, biotin *N*-hydroxysuccinimide ester; sulpho-NHS, sulphosuccinimidobiotin; BNHS-LC, sulphosuccinimido-6-(biotinamido)hexanoate; DBB, *p*-diazobenzoylbioctin; photo, photobiotin acetate; BH, biotin hydrazide; NBT, Nitro Blue Tetrazolium chloride; BCIP, 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt.

* Present address: Section on Immunology, National Institute on Alcohol Abuse and Alcoholism, 12501 Washington Avenue, Rockville, MD 20852, U.S.A.

† To whom correspondence should be addressed.

1987; Manalan & Klee, 1987; Wei *et al.*, 1988), covalent attachment of phenothiazines (Newton *et al.*, 1985; Jarrett, 1986; Faust *et al.*, 1987; Newton & Klee, 1989), dansylation (Kincaid *et al.*, 1988), biotinylation (Billingsley *et al.*, 1985, 1987, 1990; Mann & Vanaman, 1987, 1989), carbamoylation (Mann & Vanaman, 1988), photoreactive modifications (Andreasen *et al.*, 1981; O'Neil *et al.*, 1989; Harrison *et al.*, 1989) and mutational analysis of genetically modified CaMs (Wang *et al.*, 1987; Putkey *et al.*, 1988; Persechini & Kretsinger, 1988; VanBerkum *et al.*, 1990). In general, such results support the concept that individual domains of CaM are responsible for interaction with specific target proteins.

In the case of biotinylated CaM, avidin or streptavidin can be attached to CaM before interactions with target enzymes. Since avidin is a large (64 kDa) tetrameric glycoprotein, avidin-biotinyl-CaM complexes can serve to delimit regions of CaM that are sterically hindered with respect to target protein binding. Previously, we described the preparation of CaM modified with biotin *N*-hydroxysuccinimide ester (BNHS; Billingsley *et al.*, 1985, 1987, 1990). Sequence analysis with the use of radiolabelled BNHS showed that this derivative preferentially modifies Lys-94 (Mann & Vanaman, 1987). BNHS-CaM is biologically active in several CaM-dependent enzyme assays (Billingsley *et al.*, 1985, 1987; Mann & Vanaman, 1987, 1989), and can be used to identify CaM-BPs immobilized to nitrocellulose blots (Billingsley *et al.*, 1985, 1987, 1990). In order to determine whether other biotinyl derivatives of CaM are useful in studies of CaM-protein interactions, a series of biotinylated CaM congeners were prepared and their biological properties characterized.

EXPERIMENTAL

Materials

Electrophoresis supplies and organomercurial-agarose were purchased from Bio-Rad Laboratories, and molecular-mass standards were purchased from Pharmacia-LKB. Sulphosuccinimidobiotin (sulpho-NHS), biotin hydrazide (BH) and sulphosuccinimido-6-(biotinamido)hexanoate (BNHS-LC) were purchased from Pierce Chemical Co. *p*-Diazobenzoylbicytin (DBB) was obtained from Calbiochem, and photobiotin acetate (photo) was from Research Organics. Avidin was obtained from STC Laboratories. Alkaline phosphatase and leupeptin were purchased from Boehringer-Mannheim, and chromagens, Nitro Blue Tetrazolium chloride (NBT) and 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt (BCIP) were from Amresco. Biotin, 2-mercaptoethanol, snake-venom nucleosidase (*Americana crocus*) and phenylmethanesulphonyl chloride were obtained from Sigma Chemical Co. Nitrocellulose (0.22 μ m pore size) was from Schleicher and Schuell. CNBr-activated Sepharose 4B, phenyl-Sepharose, Sephadex G-25, QAE-Sephadex and DEAE-Sephadex A-25 were purchased from Pharmacia-LKB. CaM was purified from bovine testis (Groff's Meats, Elizabethtown, PA, U.S.A.) as previously described (Kincaid & Coulson, 1985; Kincaid *et al.*, 1988).

Methods

Biotinylation of CaM. After purification, CaM was dialysed overnight at 4 °C in 100-fold excess of coupling buffer (0.1 M-sodium bicarbonate buffer, pH 8.0, containing 2 mM-CaCl₂, 150 mM-NaCl and 1 mM-MgCl₂); purity of CaM (routinely > 95%) was assessed by using u.v.-absorption spectroscopy (220–340 nm) and SDS/PAGE. Five biotinyl derivatives of CaM were prepared, as follows.

(1) Sulpho-CaM. Sulpho-NHS reacts with free amino groups and is a more water-soluble analogue of BNHS. Sulpho-NHS

was dissolved in anhydrous dimethyl sulphoxide (1 mg in 50 μ l) and added to Ca²⁺-liganded CaM (2.0 mg/ml in coupling buffer) at a 10:1 molar ratio. After 2 h at 23 °C, unincorporated sulpho-NHS was removed via chromatography on Sephadex G-25 columns (PD-10) or overnight dialysis at 4 °C in 100-fold excess of coupling buffer.

(2) BNHS-LC-CaM. BNHS-LC reacts with free amino groups. This biotinyl derivative contains an extended carbon chain between the *N*-hydroxysuccinimide and the biotinyl group. Reaction conditions for CaM modification were identical with those described above.

(3) DBB-CaM. DBB can react with histidine and/or tyrosine residues. DBB was dissolved in dimethyl sulphoxide (2 mg/15 μ l) and activated on ice by the addition of 85 μ l of 1 M-HCl. After 5 min, 40 μ l of 0.112 M-NaNO₃ was added, and 5 min later the reaction was quenched with 100 μ l of 1 M-NaOH and 1750 μ l of coupling buffer. A 1 ml portion of Ca²⁺-liganded CaM (2 mg/ml) was allowed to react with 500 μ l of the activated DBB mixture (DBB/CaM molar ratio 10:1) for 90 min at 23 °C. Unincorporated DBB was removed as described above.

(4) Photo-CaM. The aryl azide of photobiotin acetate reacts with nucleophilic sites on proteins. Photobiotin acetate was dissolved in water in subdued light, and added (photobiotin acetate/CaM molar ratio 10:1) to Ca²⁺-liganded CaM (2 mg/ml dissolved in coupling buffer). The reaction mixture was kept at 4 °C by placing the reaction tube in an ice bath, which was placed 10 cm from a broad-spectrum GE sunlamp. The sample was irradiated for 10 min, and unincorporated photobiotin acetate was removed as described above.

(5) BH-CaM. BH modifies carboxy groups of acidic amino acid residues. CaM (2 mg/ml) was adjusted to pH 4.8 with conc. HCl (4 μ l/ml of CaM), and BH (6 mg/ml of dimethyl sulphoxide) was added (BH/CaM molar ratio 200:1). The coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (Sigma Chemical Co.) was added at a 10:1 molar ratio (carbodi-imide/CaM) and the reaction mixture was incubated overnight at 23 °C. Unincorporated BH was removed as described above.

Alkaline phosphatase and molecular-mass standards were biotinylated with the BNHS-LC biotinylating reagent (10:1 molar ratio) as previously described (Polli *et al.*, 1990). Alkaline phosphatase retained biological activity when biotinylated by means of this protocol.

SDS/PAGE, electroblotting and CaM overlay procedures. One-dimensional SDS/PAGE was performed with the use of a previously described protocol (O'Farrell, 1975). Pyronin Y (0.001%) was added as a tracking dye. Resolved proteins were either stained with Coomassie Brilliant Blue R-250 or electroblotted on to nitrocellulose (Towbin *et al.*, 1979). Proteins of 30–150 kDa were electroblotted at 50 V for 3–4 h. CaM derivatives were transferred for 2 h, and blots were immediately fixed in 10% (v/v) acetic acid/25% (v/v) propan-2-ol for 10 min. Blots were washed in Tris-buffered saline (150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4) containing 1 mM-CaCl₂ for 10 min. Non-specific binding sites were blocked with a solution of 5% non-fat dried milk (Carnation) in Tris-buffered saline. CaM-BPs were detected by incubating blots with biotinyl-CaM derivatives as described previously (Billingsley *et al.*, 1985, 1987, 1990). CaM-BPs were detected by using preformed avidin-biotinylated alkaline phosphatase complexes and the NBT/BCIP chromogen system. Biotinylated CaM derivatives immobilized to nitrocellulose were detected by using biotinylated alkaline phosphatase and the NBT/BCIP chromogen system.

Assay of CaM-dependent 3',5'-(cyclic)nucleotide phosphodiesterase activity. Rat neocortical cytosol depleted of CaM was

used as a source of CaM-dependent 3',5'-(cyclic)nucleotide phosphodiesterase (CaM-PDE). Neocortex was isolated from adult male Sprague-Dawley rats, homogenized in ice-cold buffer A (0.25 M-sucrose in 10 mM-Hepes, pH 7.4, containing 1 mM-EGTA, 1 mM-EDTA, 100 μ M-leupeptin and 100 μ M-phenyl-methanesulphonyl fluoride) and centrifuged at 37000 *g* for 15 min. The resulting supernatant (S2) was centrifuged at 100000 *g* for 30 min. NaCl was added to the high-speed supernatant (final concentration 0.25 M) and the cytosolic fraction chromatographed over DEAE-Trisacryl (IBRF Reactifs) previously equilibrated in buffer A containing 0.25 M-NaCl. The void fraction was collected and used as a source of CaM-PDE. Protein concentration was determined by the method of Bradford (1976).

CaM-PDE assays were completed as described by Kincaid & Manganiello (1988). Samples for determination of enzyme activity were incubated with various concentrations of native or modified CaM in 50 mM-Bes, pH 7.2, containing 1 mM-MgCl₂, 50 μ M-cyclic [³H]AMP (35 Ci/mmol; New England Nuclear) and either 2 mM-EGTA (CaM-independent activity) or 2 mM-CaCl₂ (CaM-dependent activity) for 10 min at 30 °C. Reactions were terminated by adding 100 μ l of 0.25 M-HCl. Samples were neutralized with 100 μ l of 0.25 M-NaOH/0.1 M-Tris/HCl, pH 8.0, and free nucleoside was generated by incubation with 100 μ l of snake-venom nucleosidase (2 mg/ml) for 20 min at 30 °C. Reactants were separated by means of DEAE-Sephadex A-25 chromatography, and 5'-[³H]AMP production was measured by liquid-scintillation spectrometry. Results were expressed as pmol of 5'-AMP/min per μ g of protein; CaM-stimulated activity was determined as the difference between Ca²⁺/CaM-stimulated and basal (EGTA) activity. Each reaction was performed in triplicate.

Assay of CaM-dependent protein kinase activity. CaM-dependent protein kinase II (CaM-kinase II) activity was determined by using the CaM-depleted neocortical cytosolic fraction described above as a source of CaM-kinase II enzyme and minor modifications of previously published procedures (Goldenring *et al.*, 1984; Kelly *et al.*, 1987). CaM-kinase II activity was determined within 2–3 h of isolation of the cerebral cortex. Each incubation mixture contained 150 μ g of protein in 10 mM-Hepes, pH 7.4, containing 1 mM-MgCl₂ and 20 μ M- γ -[³²P]ATP (2 μ Ci/tube; 3000 Ci/mmol; Amersham International) in a final volume of 100 μ l. Basal phosphorylation was determined in the presence of 5 mM-EGTA, and CaM-stimulated activity was determined in the presence of 5 mM-CaCl₂ and various concentrations of biotinyl-CaM derivatives. Reactions were terminated after 3 min at 30 °C by the addition of SDS/PAGE sample buffer [100 mM-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.001% Pyronin Y], the mixtures were heated for 5 min at 95 °C and samples (75 μ g of protein/lane) were subjected to SDS/10% PAGE. Resolved gels were stained with Coomassie Brilliant Blue R-250, then dried, and autoradiography was completed at –70 °C with Kodak XAR film and Cronex enhancement screens.

Construction of affinity columns. CaM-Sepharose 4B was prepared from CNBr-activated Sepharose at a substituent ratio of 2 mg of CaM/ml of gel. Avidin-Sepharose 4B was prepared from CNBr-activated Sepharose at a concentration of 3 mg of avidin/ml of gel. Coupling efficiencies were determined by u.v.-absorption spectroscopy, and routinely ranged between 80 and 90%.

A series of affinity columns were constructed by coupling the biotinyl-CaM derivatives to avidin-Sepharose. Avidin-agarose was incubated with a 5-fold excess of biotinyl-CaM derivative

for 2 h at 23 °C. Unbound biotinyl-CaM derivative was removed by extensive washing of the column with Tris-buffered saline containing 1 mM-CaCl₂ and 1 mM-MgCl₂. A column (1.0 ml total) was constructed for each biotinyl-CaM derivative.

CaM-depleted neocortical cytosol was dialysed against Tris-buffered saline containing 1 mM-CaCl₂ and 1 mM-MgCl₂ and used as a source of CaM-BPs. After loading of cytosol on to biotinyl-CaM-avidin-Sepharose affinity columns, columns were washed with Tris-buffered saline containing 1 mM-CaCl₂ and 1 mM-MgCl₂. CaM-BPs were eluted with Tris-buffered saline containing 5 mM-EGTA. Individual 500 μ l fractions were collected, the absorbance at 280 nm was measured and CaM-BPs were determined by the use of CaM overlays (with BNHS-LC-modified CaM as the probe). Coupling of biotinyl-CaM derivatives to each avidin-Sepharose column was verified by boiling a portion of the biotinyl-CaM-avidin-Sepharose in SDS/PAGE sample buffer, followed by SDS/PAGE, blotting and incubation with biotinylated alkaline phosphatase and the NBT/BCIP chromogen system.

Hydrophobic interactions of biotinyl-CaM derivatives. Hydrophobic interaction of the biotinyl-CaM derivatives was examined by phenyl-Sepharose chromatography. Each derivative was chromatographed on a 2 ml phenyl-Sepharose column equilibrated with Tris-buffered saline containing 1 mM-CaCl₂ and 1 mM-MgCl₂. Biotinyl derivatives were eluted with Tris-buffered saline containing 5 mM-EGTA. Individual 500 μ l fractions were collected and the absorbance at 280 nm was measured.

H.p.l.c. of biotinyl-CaM derivatives. Biotinyl-CaM derivatives were analysed and purified by both reverse-phase h.p.l.c. (C₁₈, Vydac) and f.p.l.c. (Mono Q; Pharmacia). For f.p.l.c. the ion-exchange column was developed with a linear gradient of 0–0.5 M-NaCl in 10 mM-Tris/HCl buffer, pH 7.4, at a flow rate of 1 ml/min. Peak fractions were analysed by scanning u.v. spectroscopy (340–220 nm); selected peaks were immobilized on nitrocellulose, and biotinyl-peptides were detected by using avidin-alkaline phosphatase chromogen systems.

Fractions of biotinylated CaM were also subjected to reverse-phase h.p.l.c. with a Waters 600 series pump and detection at 220 nm. The C₁₈ column was developed with a linear gradient of 0–60% (v/v) acetonitrile in water. In one series of experiments, biotinyl-CaM derivatives were subjected to tryptic digestion followed by mapping via reverse-phase h.p.l.c. with a C₁₈ column and a 0–60% (v/v) acetonitrile gradient. Biotinyl-CaM (100 μ g) was digested with trypsin (0.2 μ g) in coupling buffer, pH 8.0, for 90 min at 37 °C. Tryptic fragments were separated by using a C₁₈ column as described above. Biotinyl-CaM derivatives were digested in the presence of either 1 mM-CaCl₂ or 2 mM-EGTA, as previously described (Newton & Klee, 1989).

RESULTS

Preparation of biotinyl-CaM derivatives

A series of biotinylated CaM derivatives were prepared by using the reagents shown in Table 1. These biotinylating reagents were chosen because they are directed to different amino acid target sites and vary in spacer-chain length between the reactive group and biotinyl moiety. Biotinylation of CaM with BH and carbodi-imide coupling (which modifies acidic amino acid residues) yielded a CaM derivative that was biologically inactive in enzyme assays and blot overlays (results not shown). Therefore BH derivatives of CaM were not further characterized.

The extent of biotinylation of CaM was estimated by incubating each derivative with an excess of avidin-Sepharose. Biotinyl-

Table 1. Structures of biotinylating reagents and their sites of protein modification

Biotinylating reagent	Site modified
<p>Biotin <i>N</i>-hydroxysuccinimide ester (BNHS)</p>	Lysine residues, free amino groups
<p>Sulphosuccinimidobiotin (sulpho-NHS)</p>	Lysine residues, free amino groups
<p>Sulphosuccinimido-6-(biotinamido)-hexanoate (BNHS-LC)</p>	Lysine residues, free amino groups
<p><i>p</i>-Diazobenzoylbiocytin (DBB)</p>	Histidine/tyrosine residues
<p>Photobiotin acetate (photo)</p>	Nucleophilic sites
<p>Biotin hydrazide (BH)</p>	Aspartic acid/glutamic acid residues

Fig. 1. Extent of biotinylation of CaM

After biotinylation each CaM derivative was incubated with avidin–Sepharose. Key: C, CaM; S, sulfo-CaM; B, BNHS-LC-CaM; D, DBB-CaM; P, photo-CaM. The mixture of avidin–Sepharose and CaM derivative was rocked for 2 h at 4 °C and the CaM derivative–avidin–Sepharose complex was pelleted by centrifugation. SDS/PAGE sample buffer was added to the resulting supernatant (s) and pellet (p), and fractions were boiled and subjected to SDS/PAGE. Four times the volume of the supernatant was loaded compared with the pellet sample. Protein was detected with both Coomassie Brilliant Blue (a) and the biotinylated alkaline phosphatase/NBT/BCIP chromogen system (b). Std, molecular-mass standards. The results demonstrate that more than 90 % of the CaM was biotinylated.

CaM–avidin–Sepharose complexes were separated from unmodified CaM by centrifugation at 13000 *g*. Supernatants were removed and prepared for SDS/PAGE; the pelleted resin was washed with Tris-buffered saline and resuspended in a volume of SDS/PAGE sample buffer equal to the volume of supernatant. All samples were heated for 5 min at 95 °C to disrupt the biotin–avidin interaction, and portions from both the avidin matrix (25 μ l) and supernatant (100 μ l) were subjected to SDS/PAGE. Biotinyl–CaMs immobilized on nitrocellulose were detected by using avidin–alkaline phosphatase, and biotinyl–CaMs in SDS/PAGE gels were detected by staining with Coomassie Brilliant Blue.

The results in Fig. 1 demonstrate that more than 90 % of the biotinyl–CaM derivatives was retained on the avidin–Sepharose columns, indicating that most CaM molecules were biotinylated. Stoichiometry of biotinylation could not be determined in this experiment. The major biotinylated CaM species had a molecular mass of 17 kDa, similar to that of native CaM. Sulpho–CaM had several smaller biotinyl–peptides, which were detected by alkaline phosphatase but not by Coomassie Brilliant Blue staining. These bands may represent a CaM derivative having more than one biotin modification, different Ca²⁺-binding conformational states or minor proteolytic products. Incubation of this derivative with 2 mM-EGTA before electrophoresis results in the formation of only one detectable electrophoretic band (J. W. Polli & M. L. Billingsley, unpublished work).

Chromatographic characterization of biotinyl–CaM derivatives

F.p.l.c. was used to characterize native CaM and biotinyl–CaM derivatives. Native CaM was eluted as a single well-defined peak from Mono Q f.p.l.c. columns (0.4 M-NaCl). Each biotinylated derivative was eluted as a single peak (Table 2). Spectrometric analysis of each peak confirmed that the derivative corresponded to the original material, and slot blots confirmed that biotin was incorporated into each derivative.

Reverse-phase h.p.l.c. was used to map tryptic-digest profiles of native and biotinylated CaMs (Fig. 2). Native CaM yielded several distinct peptides, eluted at different acetonitrile concentrations. The profile of peaks was altered when EGTA was included in the tryptic digestion. Each derivative gave a characteristic tryptic-digest profile, suggesting that each modification affected both the tryptic digestion and elution of modified tryptic fragments. BNHS-LC-CaM and sulfo-CaM gave similar

Table 2. Elution characteristics of native and modified CaM derivatives from Mono Q columns

Each peak was subjected to scanning u.v. spectroscopy and compared with scans performed before chromatography of the modified CaM.

Derivative	F.p.l.c. elution (mM-NaCl)
Native CaM	400
Sulpho–CaM	500
BNHS-LC–CaM	150
DBB–CaM	500
Photo–CaM	400

tryptic-digestion patterns in both the presence and the absence of Ca²⁺. DBB–CaM and photo–CaM showed different profiles when digested in the presence of Ca²⁺.

Differential activation of CaM–PDE activity by biotinyl–CaM derivatives

A high-speed CaM-depleted preparation of rat neocortical cytosol was used to determine activation of CaM–PDE by each biotinyl–CaM derivative. Equimolar amounts of biotinylated and native CaM were added to CaM–PDE reactions. Separate reactions were performed in which CaM derivatives were preincubated for 10 min at 4 °C with a 10-fold molar excess of avidin before addition to the enzyme assay.

Native CaM stimulated CaM–PDE activity, with half-maximal stimulation at 25 nM–CaM (Fig. 3). Each biotinyl–CaM was able to activate CaM–PDE activity; however, 2–5-fold more biotinyl derivative was needed to achieve maximal CaM–PDE stimulation. When avidin was preincubated with the assay components, a strong inhibition of CaM–PDE activity was observed for the sulfo–CaM derivative. Similar preincubations of other CaM derivatives with avidin had little effect on the activation of CaM–PDE.

Differential activation of CaM–kinase II by biotinyl–CaM derivatives

A series of reactions were performed to examine the effect

Fig. 2. Tryptic-digestion patterns of biotinyl-CaM derivatives

Each panel illustrates the chromatographic profile of biotinylated and native calmodulins following tryptic digestion (see the Experimental section). Approx. 10 μ g of each derivative was digested in the presence of Ca^{2+} or EGTA. Samples were eluted from the C_{18} column with a linear gradient of acetonitrile (-----).

of different biotinylations of CaM on the activation of CaM-kinase II. As shown in Fig. 4, native CaM stimulated the phosphorylation of several brain cytosolic proteins in a concentration- and Ca^{2+} -dependent manner. Prominent phosphorylation of 50 kDa and 60 kDa peptides were observed, corresponding to the autophosphorylation of the major subunits

of CaM-kinase II. All biotinyl derivatives stimulated Ca^{2+} /CaM-dependent phosphorylation. However, sulpho-CaM did not produce maximal stimulation. Native CaM, DBB-CaM, BNHS-LC-CaM and photo-CaM were unaffected by preincubation with avidin; however, sulpho-CaM-avidin was unable to stimulate CaM-kinase II autophosphorylation. This result suggested that

Fig. 3. Dose-response curves and the effects of avidin on CaM-PDE activation by biotinyl-CaM derivatives

Adult cortex cytosol was depleted of endogenous CaM by passing the sample over a DEAE-Sephadex column and collecting the 0.25 M-NaCl fraction. The assay was initiated by the addition of cyclic [^3H]AMP and activity measured by the production of 5'-AMP/min per μg of protein. Results are reported as percentages of native CaM activity. Preincubation with avidin was included to determine whether the presence of avidin had any effect on enzyme activity. □, Activation by native CaM; ◆, activation by biotinyl-CaM derivative.

Fig. 4. Dose-response curves and the effects of avidin on CaM-kinase II activation by biotinyl-CaM derivatives

Adult cortex cytosol was depleted of endogenous CaM by passing the sample over a DEAE-Sephadex column and collecting the 0.25 M-NaCl fraction. Tissue (150 μg) was added to tubes containing different concentrations of biotinyl-CaM derivatives. Key: C, CaM; S, sulpho-CaM; B, BNHS-LC-CaM; D, DBB-CaM; P, photo-CaM. Several tubes contained a 10-fold molar excess of avidin, allowing the effects of avidin on enzyme activation to be determined. [$\gamma\text{-}^{32}\text{P}$]ATP was added to initiate the autophosphorylation of CaM-kinase II. Proteins were separated by SDS/PAGE, and the gels were dried and subjected to autoradiography.

Fig. 5. Hydrophobic interactions of biotinyl-CaM derivatives with phenyl-Sepharose

Biotinyl derivatives of CaM were chromatographed on 2 ml phenyl-Sepharose columns equilibrated with Tris-buffered containing 2 mM- CaCl_2 . CaM derivatives were eluted by the addition of 5 mM-EGTA to the buffer. Protein concentrations in fractions were quantified by u.v.-absorption spectrometry (280 nm). $\square$, Native CaM; $\blacklozenge$, biotinyl-CaM derivative.

preincubation of sulpho-CaM with avidin produced a sterically hindered complex unable to interact with CaM-kinase II.

Chromatographic characterization of biotinyl-CaM derivatives

CaM interacts with phenyl-Sepharose in a Ca^{2+} -dependent manner. A series of chromatographic experiments with phenyl-Sepharose were completed to determine whether biotinyl derivatives of CaM exhibited altered binding to this hydrophobic matrix. As shown in Fig. 5, native CaM bound to phenyl-Sepharose in the presence of Ca^{2+} and was eluted with 5 mM-EGTA. Each biotinyl derivative exhibited a different elution profile. Most of the sulpho-CaM was not retained on the phenyl-Sepharose matrix, whereas a considerable proportion of BNHS-LC-CaM and DBB-CaM interacted with the column in a Ca^{2+} -dependent manner. Virtually all of the photo-CaM was retained on phenyl-Sepharose. The results of this experiment demonstrate that biotinylation of certain amino acid residues alters the interaction of modified CaM with phenyl-Sepharose.

Directed affinity chromatography with biotinyl-CaM-avidin-Sepharose columns

Previous experiments suggested that biotinylated CaM derivatives exhibited differential behaviour with respect to activation of target enzymes and the effect of avidin preincubation on such activation. A series of chromatographic experiments were performed with biotinyl-CaM derivatives immobilized on avidin-Sepharose. Such 'oriented' affinity chromatography may result in the selective isolation of different CaM-BPs. Fig. 6(a) shows the elution profile and CaM-overlay analysis of proteins from CaM-depleted rat neocortical cytosol isolated on a native CaM-

Sepharose column. Major CaM-BPs were observed at 50, 58, 60 and 75 kDa. No CaM-BPs were eluted from avidin-Sepharose incubated with native CaM (results not shown).

When sulpho-CaM was coupled to avidin-Sepharose, few CaM-BPs were detected on elution with EGTA (Fig. 6b). This result suggests that the orientation of sulpho-CaM on the avidin matrix was unfavourable for interaction with target proteins and is consistent with other results. The elution profile and CaM overlay of proteins isolated from the BNHS-LC-CaM-avidin-Sepharose column were indistinguishable from CaM-Sepharose (Fig. 6c). The major proportion of CaM-BPs bound to a DBB-CaM-avidin-Sepharose column (Fig. 6d), whereas a much smaller proportion of CaM-BPs bound to a photo-CaM-avidin-Sepharose column (Fig. 6e).

In order to demonstrate that biotinyl-CaM was bound to the avidin-Sepharose matrix and to confirm that columns had similar substituent ratios, a portion of each column was boiled in SDS/PAGE sample buffer and analysed by using SDS/PAGE and blotting. As shown in Fig. 7, similar amounts of biotinyl-CaM were released from each of the affinity columns. Native CaM was not removed from CaM-Sepharose when similarly processed. It was concluded that efficiencies of attachment to avidin-Sepharose were similar for each derivative.

DISCUSSION

Biotinyl-CaM derivatives were prepared and characterized with reference to the extent of biotinylation, ability to activate several CaM-dependent enzymes, and interaction with different

Fig. 6. Biotinyl-CaM-avidin-Sepharose chromatography

Biotinyl-CaM derivatives were incubated with avidin-Sepharose to make biotinyl-CaM-avidin-Sepharose affinity columns. Adult rat neocortex cytosol depleted of CaM was chromatographed over native CaM (CaM bound to CNBr-activated Sepharose directly) (a), sulpho-CaM (b), BNHS-LC-CaM (c), DBB-CaM (d) or photo-CaM (e) affinity columns. The columns were washed extensively with Tris-buffered saline containing 2 mM- CaCl_2 (lane 1, Coomassie Brilliant Blue stain; lane 3, BNHS-LC-CaM overlay), and CaM-BPs were collected by the addition of 5 mM-EGTA to the buffer (lane 2, Coomassie Brilliant Blue stain; lane 4, BNHS-LC-CaM overlay). Std, molecular-mass standards. Protein concentrations in fractions were quantified by u.v.-absorption spectrometry (280 nm) followed by SDS/PAGE. Bands were detected by Coomassie Brilliant Blue staining or BNHS-LC-CaM overlay as described.

Fig. 7. Recovery of biotinyl-CaM derivatives from avidin-Sepharose columns

CaM (C) and the biotinyl-CaM derivatives sulpho-CaM (S), BNHS-LC-CaM (B), DBB-CaM (D) and photo-CaM (P) used in the oriented affinity-column experiment (Fig. 5) were tested to see if the coupling efficiencies were similar and whether biotinylated CaM could be recovered from the column. Part of each column was added to a portion of SDS/PAGE sample buffer, boiled and subjected to SDS/PAGE. Std, molecular-mass standards; NC, native calmodulin. Proteins were detected in SDS/polyacrylamide gels by staining with Coomassie Brilliant Blue (a) or on nitrocellulose blots by incubation with biotinylated alkaline phosphatase and the NBT/BCIP chromogen system (b).

chromatographic materials. A summary of the biological properties and possible sites of modification are shown in Table 3. Sulpho-CaM, a short-spacer-arm biotinylating agent that modifies free amino groups (lysine residues), was able to activate CaM-PDE and CaM-kinase II; however, preincubation of sulpho-CaM with avidin blocked the activation of these enzymes. Sulpho-NHS is a water-soluble analogue of BNHS, and the only difference in structure is the addition of a sulphate group on the succinimide (Table 1); therefore these agents are presumably directed towards the same target sites. Mann & Vanaman (1987) have previously identified Lys-94 as the site of CaM modified by BNHS. This finding, in conjunction with previous work demonstrating that the u.v.-absorption spectra of BNHS-CaM and sulpho-CaM are identical (Billingsley *et al.*, 1990), suggests that sulpho-CaM is also modified at Lys-94. Further, the results presented here demonstrating that preincubation of sulpho-CaM with avidin abolishes CaM-PDE activation concur with the finding that the addition of avidin inhibited BNHS-CaM activation of CaM-PDE (Mann & Vanaman, 1987, 1989).

Even though these derivatives (sulpho-CaM and BNHS-CaM) bind and activate CaM-PDE, they are unable to detect CaM-PDE immobilized on nitrocellulose (Billingsley *et al.*, 1987; Mann & Vanaman, 1987). This is most probably due to the biotin being shielded from avidin when these biotinyl derivatives bind to CaM-PDE immobilized on nitrocellulose. Therefore avidin, used for detection in conjunction with alkaline phosphatase, cannot bind to the biotin residue owing to the steric hindrance of the enzyme. Sulpho-CaM was able to activate CaM-kinase II; however, as was seen with CaM-PDE, preincubation of sulpho-CaM with avidin abolished CaM-kinase II activation. CaM-kinase II immobilized on nitrocellulose is detectable with these biotinyl-CaM derivatives (Polli *et al.*, 1990; Billingsley *et al.*, 1990).

BNHS-LC is an analogue of sulpho-NHS and BNHS containing an extended spacer arm (2.25 nm compared with 1.35 nm). BNHS-LC-CaM was able to activate both CaM-PDE and CaM-kinase II. Preincubation with avidin had no effect on BNHS-LC-CaM activation of CaM-PDE or CaM-kinase II. This suggests that the extended spacer arm is long enough to allow avidin access to the biotin moiety. Chromatographic data

Table 3. Properties and probable sites of biotinylation of CaM derivatives

Derivative	Sites of biotin	CaM-PDE	CaM-kinase II	Effect of avidin
Native CaM	None	++++	++++	None
BNHS-LC-CaM	Lys-94 Lys-77	+++	+++	None
Sulpho-CaM	Lys-94 Lys-77? Lys-30?	++	++	Inhibition
DBB-CaM	His-107 Tyr-138	++++	++++	None
Photo-CaM	Lys-94?	+++	+++	None

revealed that both sulpho-CaM and BNHS-LC-CaM have diminished binding to phenyl-Sepharose, demonstrating that these modifications alter the structure within the hydrophobic region of CaM. 'Oriented' affinity chromatography demonstrated a decreased ability of sulpho-CaM to bind CaM-BPs in solution. BNHS-LC-CaM was able to bind a set of CaM-BPs identical with those bound by native CaM. This further demonstrates that such an 'oriented' CaM molecule can only bind a select population of CaM-BPs. The extended spacer arm of BNHS-LC-CaM frees CaM so that it can bind a larger population of CaM-BPs in solution. Previous work has also shown that BNHS-CaM, BNHS-LC-CaM and sulpho-CaM recognize a similar pattern of CaM-BPs immobilized on nitrocellulose (Billingsley *et al.*, 1987, 1990).

Several other biotinyl derivatives of CaM were prepared and characterized. BH, which modifies acidic amino acid residues, yielded a biologically inactive molecule. Inactivation of CaM is most probably due to extensive modification of glutamic acid and aspartic acid residues resulting in the disruption of Ca^{2+} -binding and tertiary structure.

DBB reacts with tyrosine and/or histidine residues and contains an extended spacer arm. CaM contains two tyrosine residues (residues 99 and 138) and one histidine residue (residue 107). DBB-CaM was able to activate CaM-PDE and CaM-kinase II either in the presence or in the absence of avidin. Chromatographic experiments demonstrated that DBB-CaM binds to phenyl-Sepharose in a Ca^{2+} -dependent manner. When DBB was bound to avidin-Sepharose, a population of CaM-BPs was isolated similar to that isolated with the use of BNHS-LC-CaM or native CaM. Earlier work on tyrosine modification demonstrated that biotinylation of Tyr-99 yields a derivative that can activate CaM-PDE (Mann & Vanaman, 1987). Activation of CaM-PDE by the Tyr-99 derivative was blocked by preincubation with avidin. Modification of Tyr-138 yielded a derivative that activates CaM-PDE in the presence and in the absence of avidin (Mann & Vanaman, 1987). Therefore it is concluded that DBB-CaM is modified at either Tyr-138 or His-107.

The final derivative prepared was photo-CaM. Photobiotin contains an extended spacer arm and modifies nucleophilic sites. This derivative of CaM activated CaM-PDE and CaM-kinase II in the presence and in the absence of avidin. Photo-CaM bound to phenyl-Sepharose, but had diminished binding to CaM-BPs when coupled to avidin-Sepharose. The site of modification is not known at the present time.

Results of h.p.l.c. and f.p.l.c. suggested that the *N*-hydroxy-succinimide derivatives behaved similarly, but that DBB-CaM and photo-CaM were modified in different sites. Although sequence analysis is needed to confirm the exact sites of biotinylation, such experiments are hindered by the lack of radio-

tracers for the various biotin derivatives used in this study. Several attempts to identify the biotin-containing sites were unsuccessful, in that non-radioactive biotinyl regions were not able to be detected at sufficient sensitivity following high-sensitivity automated Edman degradation (M. L. Billingsley, unpublished work).

These experiments suggest that production of several biotinyl derivatives of a given agent is a good strategy for developing biotinylated proteins for use in the study of protein-protein interactions. Although modification of a protein with a given congener may inactivate the protein, this effect can not be determined *a priori*. The *N*-hydroxysuccinimide derivatives are frequently employed in biotinylation experiments. However, these reagents can inactivate proteins such as nerve growth factor (Rosenberg *et al.*, 1986). In the case of nerve growth factor, BH was used and yielded a biologically active growth factor. Therefore utilizing a series of biotinylating reagents targeted to different protein sites should facilitate development of a biologically active derivative.

These studies point to a broader use of biotinyl-proteins. Biotinylated proteins, in conjunction with avidin, can be used to map sites of interaction between proteins. The results also demonstrate that the length of the spacer between biotin and protein is an important structural component of the biotinylating reagent. In the case of *N*-hydroxysuccinimide derivatives, the spacer decreases the steric hindrance of the protein-CaM interaction, allowing for avidin to bind to the biotin moiety. Future production of a series of further biotinylated CaM derivatives will generate a set of cellular probes to permit the study of the CaM regulatory cascade *in vivo*.

This work was supported by research grants from the International Life Sciences Institute Research Foundation and U.S. Public Health Service Grants R01-AG06377 and R01-ES05450 to M.L.B. The work was supported by a Pharmaceutical Manufacturers Association Predoctoral Fellowship to J.W.P. We thank Christine Patanow for her technical assistance.

REFERENCES

- Andreasen, T. J., Keller, C. H., LaPorte, D. C., Edelman, A. M. & Storm, D. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2782-2785
- Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R. & Cook, W. J. (1985) *Nature (London)* **315**, 37-40
- Billingsley, M. L., Pennypacker, K. R., Hoover, C. G., Brigati, D. J. & Kincaid, R. L. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7585-7589
- Billingsley, M. L., Pennypacker, K. R., Hoover, C. G. & Kincaid, R. L. (1987) *Biotechniques* **5**, 22-31
- Billingsley, M. L., Polli, J. W., Pennypacker, K. R. & Kincaid, R. L. (1990) *Methods Enzymol.* **184**, 451-467
- Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3187-3191
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Cheung, W. Y. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2253-2257
- Faust, F. M., Slisz, M. & Jarrett, H. W. (1987) *J. Biol. Chem.* **262**, 1938-1941
- Giedroc, D. P., Sinha, S. K., Brew, K. & Puett, D. (1985) *J. Biol. Chem.* **260**, 13406-13413
- Goldenring, J. R., McGuire, J. S., Jr. & Delorenzo, R. J. (1984) *J. Neurochem.* **42**, 1077-1084
- Harrison, J. K., Lawton, R. G. & Gnegy, M. E. (1989) *Biochemistry* **28**, 6023-6027
- Jarrett, H. W. (1986) *J. Biol. Chem.* **261**, 4967-4972
- Kelly, P. T., Shields, S., Conway, D., Yip, R. & Burgin, K. (1987) *J. Neurochem.* **49**, 1927-1939
- Kincaid, R. L. & Coulson, C. C. (1985) *Biochem. Biophys. Res. Commun.* **133**, 256-264
- Kincaid, R. L. & Manganiello, V. C. (1988) *Methods Enzymol.* **159**, 457-470
- Kincaid, R. L., Billingsley, M. L. & Vaughen, M. (1988) *Methods Enzymol.* **159**, 605-626
- Klevit, R. E., Blumenthal, D. K., Wemmer, D. E. & Krebs, E. G. (1985) *Biochemistry* **24**, 8152-8157
- Kretsinger, R. H., Rudnick, S. E. & Weissman, L. C. (1986) *J. Inorg. Biochem.* **28**, 289-302
- Manalan, A. S. & Klee, C. B. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **18**, 227-278
- Manalan, A. S. & Klee, C. B. (1987) *Biochemistry* **26**, 1382-1390
- Mann, D. M. & Vanaman, T. C. (1987) *Methods Enzymol.* **139**, 417-433
- Mann, D. M. & Vanaman, T. C. (1988) *J. Biol. Chem.* **263**, 11284-11290
- Mann, D. M. & Vanaman, T. C. (1989) *J. Biol. Chem.* **264**, 2373-2378
- Newton, D. L. & Klee, C. B. (1989) *Biochemistry* **28**, 3750-3757
- Newton, D., Klee, C., Woodgett, J. & Cohen, P. (1985) *Biochim. Biophys. Acta* **845**, 533-539
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007-4021
- O'Neil, K. T. & DeGrado, W. F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4954-4958
- O'Neil, K. T. & DeGrado, W. F. (1990) *Trends Neurosci.* **15**, 59-64
- O'Neil, K. T., Erickson-Viitanen, S. & DeGrado, W. F. (1989) *J. Biol. Chem.* **264**, 14571-14578
- Persechini, A. & Kretsinger, R. H. (1988) *J. Biol. Chem.* **263**, 12175-12178
- Persechini, A., Moncrief, N. D. & Kretsinger, R. H. (1989) *Trends Neurosci.* **12**, 462-467
- Polli, J. W., Patanow, C. M. & Billingsley, M. L. (1990) *Brain Res.* **53**, 62-70
- Putkey, J. A., Ono, T., VanBerkum, M. F. A. & Means, A. R. (1988) *J. Biol. Chem.* **263**, 11242-11249
- Rosenberg, M. B., Hawrot, E. & Breakfield, X. O. (1986) *J. Neurochem.* **46**, 641-648
- Stoclet, J., Gerard, D., Kilhoffer, M., Lugnier, C., Miller, R. & Schaeffer, J. (1987) *Prog. Neurobiol.* **29**, 321-364
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350-4354
- VanBerkum, M. F. A., George, S. E. & Means, A. R. (1990) *J. Biol. Chem.* **265**, 3750-3756
- Wang, J. H., Suzuki, T., Mooibroek, M., Adachi, M., Sharma, R. K. & Lam, Y. H. (1987) *Methods Enzymol.* **139**, 518-534
- Wei, Q., Jackson, A. E., Pervaiz, S., Carraway, K. L., III, Lee, E. Y. C., Puett, D. & Brew, K. (1988) *J. Biol. Chem.* **263**, 19541-19544
- Winkler, M. A., Fried, V. A., Merat, D. L. & Cheung, W. Y. (1987) *J. Biol. Chem.* **262**, 15466-15471

Received 6 August 1990/8 October 1990; accepted 16 October 1990