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Reproducible Production of Antiserum against Vertebrate Calmodulin and Determination of the Immunoreactive Site*

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Calmodulin is a small, acidic, calcium-binding protein that exhibits multiple *in vitro* biochemical activities. Although calmodulin has no known enzymatic activity, it stimulates several enzyme activities in a calcium-dependent manner. Because of its ubiquitous distribution and highly conserved structure, it has been difficult to elicit anti-calmodulin sera of useful titer. We describe here a reproducible and rapid method for producing anti-calmodulin sera. This method requires the injection of performic acid-oxidized calmodulin, but the antisera react equally well with unoxidized calmodulin. A response was elicited in 11 out of 11 rabbits using three variations of this method. Antisera titers were high enough to enable development of a quantitative radioimmunoassay using dilutions of whole sera, immunoglobulin fractions, or immunoglobulin fractions purified on calmodulin-Sepharose conjugates. For the majority of the antisera, the immunoreactive site is contained in a unique region of the calmodulin molecule. Based on the quantitative reactivity of overlapping tryptic and cyanogen bromide peptides, we propose that a major immunoreactive site is found within an 18-residue region in the COOH-terminal domain of calmodulin.

Calcium is a mediator of many cellular processes and a regulatory agent of major importance in the homeostasis of individual cells as well as whole organisms. In order to elucidate the mechanisms of calcium control and the mechanisms of action of many hormones, regulatory agents, toxins, and pharmacological agents, it is necessary to examine the cellular receptors for calcium and the means by which these receptors mediate the biological effects of calcium. Calmodulin and other members of the family of calcium-modulated proteins are major intracellular receptors for calcium (1).

Calmodulin is the name proposed (2) for a protein (3) previously referred to as modulator protein or brain troponin C-like protein. Calmodulin has multiple *in vitro* activities (for a review, see Ref. 4), but the physiological significance of a number of these activities remains obscure. However, calmodulin or calmodulin activity has been detected in most eukaryotic cells examined, and the amino acid sequence and *in vitro* activities of calmodulin are highly conserved throughout vertebrate and invertebrate species (3, 5–10). These multiple *in vitro* effects, the apparent ubiquitous distribution, and the highly conserved structure suggest that calmodulin may play a fundamental role in mediating intracellular calcium-dependent effects.

Because calmodulin is such a highly conserved protein, it has been difficult to elicit antisera of useful titer for studies of the role of calmodulin in cell function. If the antigenic sites in calmodulin were well defined in molecular terms, anti-calmodulin sera would also be useful for structure-function studies as molecular probes of functional domains. Finally, the ability to elicit rapidly and reproducibly high titer antisera would be useful in attempts to produce monoclonal antibodies against calmodulin.

We describe here a reproducible and rapid method for producing anti-calmodulin sera whose specificity appears to be confined to a unique sequence in the molecule. A preliminary communication of some of these results has been reported (11).

MATERIALS AND METHODS

Calmodulin was purified from chicken gizzard and bovine brain as described previously (6, 12). Performic acid oxidation of calmodulin was done essentially as described by Hirs (13). Briefly, performic acid reagent was prepared by adding 0.5 ml of 30% (v/v) hydrogen peroxide to 9.5 ml of formic acid (Eastman or J. T. Baker; reagent grade in glass bottles), incubating the covered solution at room temperature for 2 h, then chilling it on ice for 30 min. Vertebrate calmodulin (usually 40–50 mg) was dissolved in 1 ml of formic acid and incubated on ice for 30 min. Three ml of the performic acid reagent was added to the calmodulin and the reaction was allowed to proceed for 150 min on ice. The mixture was then diluted with water and rotary evaporated. The dried residue was redissolved in water, shell-frozen, and lyophilized. The lyophilized powder was dissolved in water at approximately a 4 mg/ml concentration and stored at −20 °C in small aliquots until injection.

As described under "Results," three different injection schedules were used. In all three schedules, injections were subcutaneous in 4–5 sites along the back of New Zealand white, *Pasteurella*-free, female rabbits. The antigen was emulsified in either complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (subsequent injections). For the studies described here, a total of 0.5–1.0 mg of vertebrate calmodulin was administered to each rabbit per injection. Blood, obtained from the ear vein, was allowed to clot at 4 °C overnight. The serum, separated by centrifugation at 900 × *g* for 15 min, was stored in small aliquots at −20 or −80 °C. Immunoglobulin fractions were prepared by the method of Harboe and Ingild (14) or Wallace *et al.* (15). Usually, serum was made 18% (w/v) in sodium sulfate, incubated at room temperature for 30 min, and centrifuged at 900 × *g* for 30 min. The resultant pellet was resuspended in 0.17 M borate-buffered saline, pH 8.0, and dialyzed overnight at 4 °C. The dialyzed material was clarified by centrifugation at 900 × *g* for 15 min and applied to a DEAE-A50 column in 0.01 M sodium phosphate, pH 7.5. The immunoglobulin fraction was eluted with two column volumes of buffer and dialyzed against phosphate-buffered saline. Affinity-based adsorption chromatography of immunoglobulins was done by application of immunoglobulin fractions to columns of calmodulin-Sepharose conjugates previously equilibrated with phosphate-buffered saline, removal of adventitiously bound material by elution with phosphate-buffered saline, then elution of antigen-adsorbed immunoglobulins with 2 M $MgCl_2$. Calmodulin-Sepharose conjugates were prepared essentially as described previously (16), except that

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coupling was performed in 0.2 M sodium bicarbonate instead of triethanolamine-HCl, and cyanogen bromide-activated Sepharose was purchased from Pharmacia and prepared according to manufacturer's instructions.

Bovine brain calmodulin was iodinated using essentially the procedure of Dorval *et al.* (17). Briefly, 10 μg of calmodulin in 0.15 M sodium phosphate, pH 7.5, was iodinated at 23 °C for 25 s using 4 μg of chloramine-T and 1 mCi of ^{125}I (Amersham; 100 mCi/ml; carrier-free). The reaction was stopped by the addition of 5 μg of sodium metabisulfite and 0.25 ml of 5% (w/v) bovine serum albumin. The reaction mixture was immediately transferred to a Sephadex G-10 column (1 × 15 cm) equilibrated in 0.15 M sodium phosphate, pH 7.5. This procedure resulted in ^{125}I-labeled calmodulin with a specific radioactivity of 700–900 Ci/mmol. Autoradiography of sodium dodecyl sulfate-polyacrylamide gels showed that the iodinated protein comigrated with unlabeled bovine brain calmodulin.

Direct radioimmunoassays were performed in RIA buffer (0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris-HCl, pH 7.6) containing 0.2% (w/v) bovine serum albumin. Assay volumes were usually 0.10 ml, although in some experiments, a 0.50-ml reaction volume was used. Reaction mixtures of 0.10 ml contained a fixed amount of ^{125}I-labeled calmodulin (1 ng; ~50,000 cpm), various concentrations of anti-calmodulin serum, and enough normal rabbit serum to give a final serum volume of 10 μl in each assay tube. After overnight incubation at 4 °C, 50 μl of goat anti-rabbit serum were added, and the incubation was continued at 4 °C for 6–24 h. RIA buffer (0.5 ml) was then added and the mixture was centrifuged at 900 × *g* for 10 min. The resultant pellet was washed with RIA buffer and recentrifuged. Radioactivity of the final pellet was determined in a Packard γ counter.

Competition radioimmunoassays were done as above by using a limiting dilution of anti-calmodulin serum (that amount determined by direct radioimmunoassay to give 50% precipitation of the added counts per min) and various concentrations of competing antigens. The anti-calmodulin serum and competing antigens were incubated at 4 °C overnight, ^{125}I-labeled calmodulin (1 ng; 50,000 cpm) was then added, and the solution was incubated at 4 °C overnight. Goat anti-rabbit serum was added and the assay mixture was incubated and processed for counting as described above.

Trypsin digestions of calmodulin were performed in 0.1 M ammonium bicarbonate containing 1 mM EGTA.[1] L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) was added to a final ratio of 1:50 (w/w) and the reaction was allowed to proceed at 37 °C overnight. The mixture was lyophilized to dryness and the powder was resuspended in 0.1% (v/v) HCl (Ultrex). Tryptic peptides were isolated by reverse phase chromatography as described previously (6, 18) on a Whatman Partisil M9 ODS-2 column (9.4 × 250 mm) using 0.1% (v/v) HCl (J. T. Baker, 12 N Ultrex) as solvent A and acetonitrile (UV grade) as solvent B.

Calmodulin was treated with cyanogen bromide by the following procedure. Calmodulin (10 mg) was dissolved in 1 ml of 70% (v/v) formic acid, and 0.1 ml of a cyanogen bromide solution (0.7 g in 1 ml of acetonitrile) was added. The mixture was incubated overnight at room temperature, rotary-evaporated to dryness, redissolved in ammonium bicarbonate, and lyophilized. The lyophilized powder was dissolved in 0.1% (v/v) HCl as above. Cyanogen bromide cleavage peptides were separated by reverse phase chromatography using the same column and buffer system as described for the tryptic peptide separation but with a different gradient elution program. The elution gradient consisted of the following time program: 0–5 min, 5% solvent B; 5–15 min, a linear increase to 20% solvent B; 15–50 min, solvent B increased to 35% and held there for 10 min; 60–65 min, solvent B increased to 45% and held there for 10 min; 75–80 min, solvent B increased to 90% and held there for 15 min. The program was ended by re-equilibrating the column in 5% solvent B.

Chymotrypsin digestions were done in 0.1 M ammonium bicarbonate using α-chymotrypsin (Worthington) at a ratio of 1:50 (w/w). Reaction mixtures were incubated overnight at 37 °C and the mixture was lyophilized to dryness. The powder was resuspended in RIA buffer and frozen at −20 °C until assayed.

Protein concentrations were determined by amino acid analysis on a Durrum D-500 instrument as described previously (6, 12). New Zealand white, *Pasteurella*-free, female rabbits were obtained from Dutchland (Denver, Pa.). Parvalbumin and S100b were gifts of R. Kretsinger (University of Virginia, Charlottesville, Va.). S100b was also purified by a modification[2] of a previously published procedure (19) for calmodulin purification. Normal rabbit serum and goat anti-rabbit serum were purchased from Cappel Laboratories (Cochranville, Pa.). Bovine serum albumin was obtained from Sigma. Distilled, deionized water was from a Darco water system (Durham, N. C.). All other chemicals were reagent grade and used without further purification or vendor preference.

RESULTS

Production of Anti-calmodulin Sera—Over the past few years, several antigen modifications have been tried in our laboratory. These procedures included: injection of calmodulin treated with sodium dodecyl sulfate, coupling of calmodulin to hemocyanin by the carbodiimide procedure, injection of dinitrophenylated calmodulin, adsorption of calmodulin to alum, and injection of large amounts of native calmodulin. A few of these procedures elicited variable and small responses, as detected by direct radioimmunoassay and as evidenced by the observation that anti-calmodulin immunoglobulins could be purified by calmodulin-Sepharose affinity chromatography. None of the above protocols elicited reproducible and high titer responses in whole sera. However, the use of performic acid-oxidized calmodulin with several injection protocols yielded anti-calmodulin sera of sufficient titer to allow assay of reactivity using dilutions of whole sera as well as antigen-adsorbed immunoglobulins.

Fig. 1 shows three injection schedules and the time course of appearance of anti-calmodulin reactivity in three representative rabbits. In schedule A, six rabbits were injected with performic acid-oxidized calmodulin on days 1, 7, 9, 13, 15, and 17, and were bled on day 27. In schedule B, two rabbits were injected with performic acid-oxidized calmodulin on days 1, 14, 16, 20, 22, and 26, and were bled on day 35. Rabbits were then boosted approximately every 2 weeks and bled 7–10 days after each boost. In schedule C, three rabbits were injected as in schedule B, except that unoxidized calmodulin was used as antigen until day 111 (*open arrow*). All subsequent injections were with performic acid-oxidized calmodulin.

The eight rabbits injected with only performic acid-oxidized calmodulin (schedules A and B) produced a response 4–5 weeks after the initial injection. There was no reactivity in any pre-immune sera. The three rabbits injected with unoxidized calmodulin (schedule C) showed little or no reactivity; however, after only two injections of performic acid-oxidized calmodulin, these rabbits produced a response. The data in Fig. 1 show the antigenic response of 3 of the 11 rabbits. However, all 11 rabbits produced anti-calmodulin sera after injection with performic acid-oxidized calmodulin. Amino acid analysis of performic acid-oxidized calmodulin demonstrated a quantitative conversion of methionine to methionine sulfone, and in one case a diminution in tyrosine content from 2 mol/mol protein to 1 mol/mol of protein. In all other respects, the composition of performic acid-oxidized calmodulin was identical with that reported previously (6) for vertebrate calmodulin.

The rabbits can be bled every 2 weeks with no adverse effects. We have found that antisera titers will decrease if booster injections are not administered regularly. Therefore, we routinely inject the rabbits with performic acid-oxidized calmodulin approximately every 4–6 weeks to sustain a high titer immune response. Five of the rabbits have been maintained for longer than a year. All 11 rabbits are healthy and are still producing antisera of comparable titer and the same molecular specificity as that described below.

Development of a Radioimmunoassay for Anti-calmodulin Sera—The anti-calmodulin sera were tested for their ability

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

[2] D. M. Watterson, unpublished.

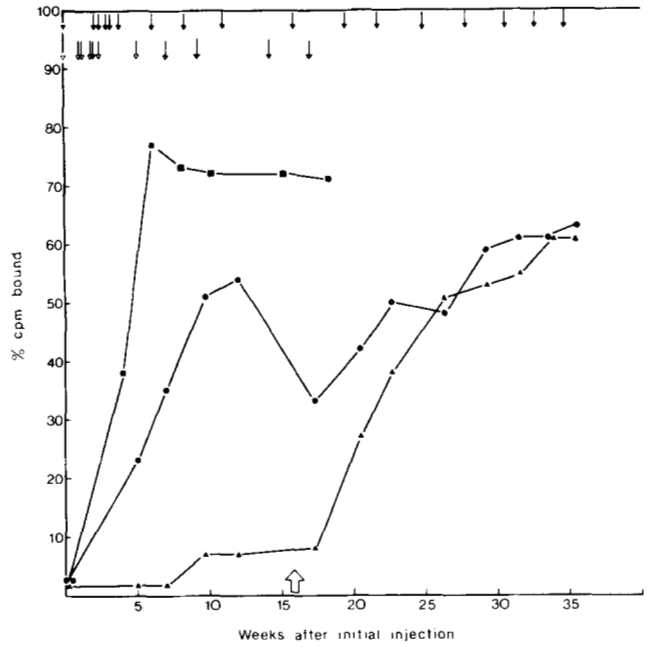

FIG. 1. **Time course of antibody production.** ■, schedule A. Rabbits were injected with performic acid-oxidized calmodulin at the times indicated by the *small open arrows.* ●, schedule B. Rabbits were injected with performic acid-oxidized calmodulin at the times indicated by the *solid arrows.* ▲, schedule C. Rabbits were injected as in schedule B, except that unoxidized calmodulin was used as antigen until the time indicated by the *large open arrow.* All subsequent injections were with performic acid-oxidized calmodulin.

to form precipitin lines in double diffusion Ouchterlony analysis (20). Under the conditions used, the sera were found to be nonprecipitating. Therefore, a radioimmunoassay that would detect precipitating and nonprecipitating antibodies was developed. We tested various methods for separating antibody-bound from free ^{125}I-labeled calmodulin. Protein A-Sepharose could be used to precipitate the antibody-bound ^{125}I-labeled calmodulin. However, the background counts per min precipitated in the absence of antiserum were high, requiring at least four buffer washes of the pellet, or washes employing detergents, to remove nonspecific binding. Therefore, a precipitation procedure using goat anti-rabbit serum was used. The background counts per min precipitated in the absence of antiserum was always less than 5%, and only two buffer washes of the pellet were required. We have found that the method of iodination of calmodulin does not significantly affect the antigenic reactivity. Our anti-calmodulin antibodies will bind to calmodulin that has been iodinated using the Bolton-Hunter reagent (21), lactoperoxidase (22), excess chloramine-T (100 μg; Ref. 23), or mild chloramine-T conditions (4 μg; Ref. 17). We routinely use the chloramine-T procedure as described under "Materials and Methods."

A direct radioimmunoassay showing the binding of ^{125}I-labeled calmodulin to anti-calmodulin serum is illustrated in Fig. 2*A*. Under the conditions used for direct radioimmunoassay, the various antisera would precipitate 60–80% of the ^{125}I-labeled calmodulin added to the reaction. There was no difference in the amount of iodinated calmodulin precipitated when assays were done in the presence of 0.1 mM $CaCl_2$, 1.0 mM EGTA, or 1.0 mM EDTA.

The data shown in Fig. 2*A* were obtained by iodinating unoxidized calmodulin, but similar results were obtained using iodinated, performic acid-oxidized calmodulin in direct radioimmunoassay. In addition, in competition radioimmunoassays using ^{125}I-labeled calmodulin, unoxidized calmodulin showed a competition curve similar to that of performic acid-oxidized calmodulin. Thus, even though the antibodies were elicited by the injection of performic acid-oxidized calmodulin, unoxidized calmodulin reacted with the antiserum as well as performic acid-oxidized calmodulin.

Upon examining the effect of different incubation conditions on competition radioimmunoassays, we observed that the same protein preparation would exhibit variable and less sensitive competition curves if EDTA were not present in the reaction mixture. Identical results were obtained with performic acid-oxidized calmodulin and with purified tryptic peptide T-1B (peptide immunoreactivity is discussed below). As noted above, these effects were never observed in direct radioimmunoassay. Therefore, in order to minimize variability and reduce artifacts of assay conditions, radioimmunoassays were routinely done in the presence of 1 mM EDTA.

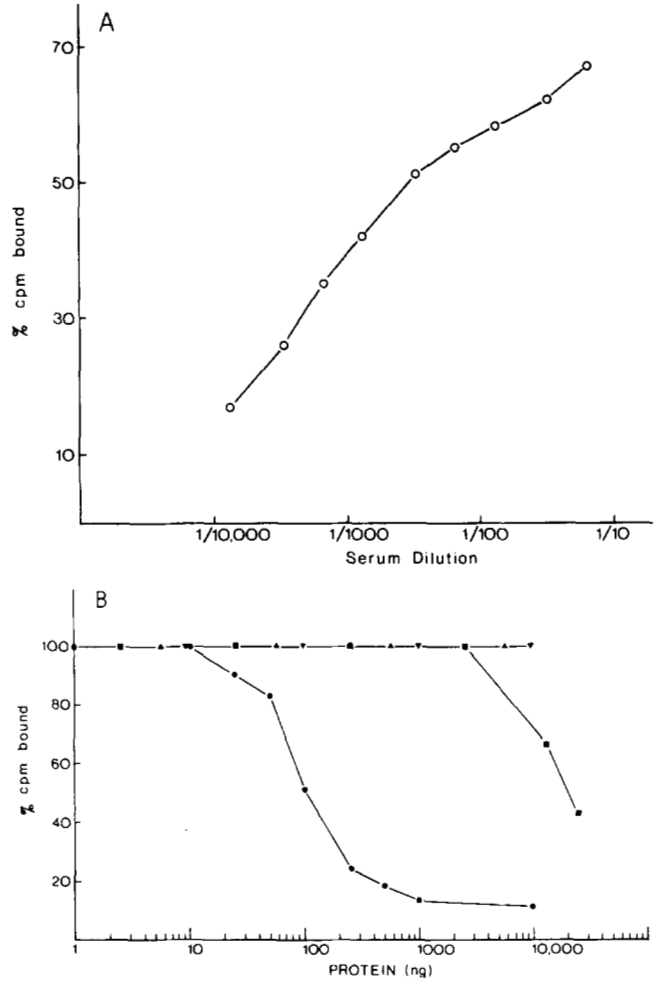

FIG. 2. **Characterization of anti-calmodulin serum by radioimmunoassay.** *A*, binding of anti-calmodulin serum to ^{125}I-labeled calmodulin in direct radioimmunoassay. Various dilutions of anti-calmodulin serum were added to a fixed concentration of ^{125}I-labeled calmodulin (1 ng; 50,000 cpm) and incubated as described in the text. The *ordinate* represents the percentage of counts per min bound. The counts per min bound in the absence of antiserum was 5%, and has not been substracted from the data shown. *B*, specificity of anti-calmodulin serum in competition radioimmunoassay. Various concentrations of bovine brain calmodulin (●), rabbit skeletal muscle troponin C (■), parvalbumin (▼), and S100b (▲) were mixed with anti-calmodulin serum (1:30 dilution) and incubated overnight at 4 °C. A fixed concentration of ^{125}I-labeled calmodulin (1 ng; 50,000 cpm) was added and the mixture was processed as described in the text. The degree of competition is expressed as a percentage of the counts per min bound in the absence of competing antigen.

Immunoreactivity of Related Proteins—The specificity of the anti-calmodulin sera was first tested by determining the ability of several structurally and functionally related proteins to compete quantitatively with ^{125}I-labeled calmodulin in radioimmunoassay. All vertebrate calmodulins examined showed competition curves similar to that of bovine brain calmodulin (Fig. 2*B*). Spinach calmodulin also quantitatively competed for antiserum prepared against vertebrate calmodulin (18). There was no reactivity with parvalbumin or brain S100b (Fig. 2*B*). Rabbit skeletal muscle troponin C showed some cross-reactivity, but approximately 400 times more protein was required for 50% competition. It is not known from these studies whether the cross-reactivity seen with troponin C at high protein concentrations is due to a minor calmodulin contamination in the troponin C preparation analogous to that found in parvalbumin preparations (24), or due to the sequence similarities between calmodulin and troponin C (3).

The data shown above were obtained using dilutions of whole anti-calmodulin sera; however, similar reactivity was found using immunoglobulin fractions and immunoglobulins purified by adsorption chromatography on calmodulin-Sepharose columns.

Determination of the Immunoreactive Site of Calmodulin—In order to characterize antiserum specificity and determine which portions of the calmodulin molecule were required for antigenic reactivity, vertebrate calmodulin was cleaved by a variety of methods and the reaction mixtures tested for immunoreactivity. A trypsin digest of calmodulin competed as well as or better than undigested calmodulin for anti-calmodulin serum (Fig. 3). While the particular experiment shown in Fig. 3 demonstrated that the digest competed slightly better than intact calmodulin, other assays showed superimposable curves for the digest and the whole protein. Ten out of 11 rabbits produced antisera which reacted with trypsin digests of calmodulin. The specificity of the one rabbit sera (number 449) which showed no reactivity with the trypsin digest of calmodulin was not investigated as part of this study.

The individual tryptic peptides were then isolated, characterized, and tested for their ability to compete with ^{125}I-labeled calmodulin for anti-calmodulin serum. Tryptic peptides encompassing the entire calmodulin molecule were tested. As shown in Fig. 3, all the immunoreactivity was contained in a single tryptic peptide, T-1B, which comprises the COOH-terminal 22 residues of the calmodulin molecule (Fig. 4) and includes most of domain 4 (for peptide nomenclature, see Ref. 3). There was no reactivity with any other tryptic peptide. Further treatment of T-1B with chymotrypsin, which preferentially cleaves peptides at the COOH-terminal side of aromatic residues, resulted in a total loss of antigenic reactivity.

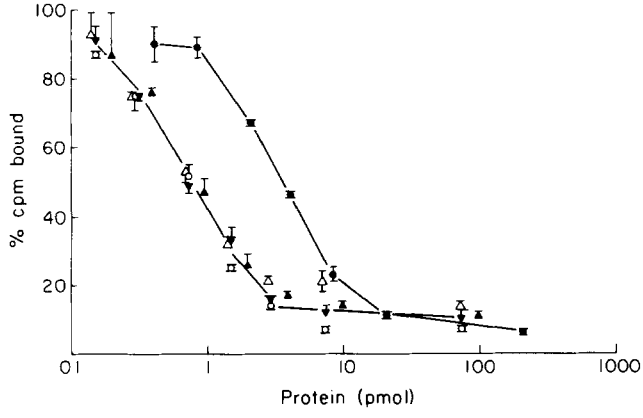

FIG. 3. **Immunoreactivity of calmodulin fragments in competition radioimmunoassay.** Various concentrations of gizzard calmodulin (●), tryptic digest (○), cyanogen bromide digest (▼), tryptic peptide T-1B (▲), and cyanogen bromide peptide 3B1 (△) were mixed with anti-calmodulin serum (1:30 dilution) and incubated overnight at 4 °C. A fixed amount of ^{125}I-labeled calmodulin (1 ng; 50,000 cpm) was added and the mixture was processed as described in the text. The degree of competition is expressed as a percentage of the counts per min bound in the absence of competing antigen.

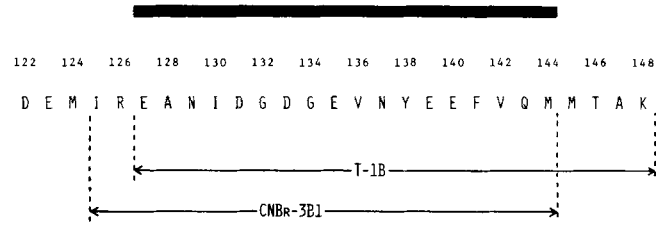

FIG. 4. **Proposed immunoreactive site of vertebrate calmodulin.** The amino acid sequence shown is that of bovine brain calmodulin (3). *Numbers* indicate the residue number in the bovine brain sequence. Cleavage positions to form tryptic peptide T-1B (residues 127–148) and cyanogen bromide peptide 3-B1 (residues 125–144) are marked with *dotted lines*. The *black bar* indicates the proposed immunoreactive site of calmodulin. *A*, Ala; *D*, Asp; *E*, Glu; *F*, Phe; *G*, Gly; *I*, Ile; *K*, Lys; *M*, Met; *N*, Asn; *Q*, Gln; *R*, Arg; *V*, Val; *Y*, Tyr.

To characterize further the immunoreactive domain, calmodulin was degraded with cyanogen bromide as described under "Materials and Methods." The reactivity of the cyanogen bromide digest was indistinguishable from that of the tryptic digest, competing as well as or slightly better than the undigested calmodulin (Fig. 3). As with the tryptic peptides, cyanogen bromide cleavage peptides were isolated, characterized, and tested for their ability to compete with ^{125}I-labeled calmodulin for anti-calmodulin serum. As shown in Fig. 3, all the immunoreactivity was contained in a single cyanogen bromide peptide, CNBr-3B1, which comprises residues 125–144 in the amino acid sequence of calmodulin and overlaps with the tryptic peptide T-1B (see Fig. 4). There was no reactivity with any other cyanogen bromide cleavage peptide.

To demonstrate directly the interaction of peptide T-1B with anti-calmodulin serum, peptide T-1B was iodinated by the same procedure used for the intact protein. The ^{125}I-labeled peptide, which had a specific radioactivity of 240 Ci/mmol, was tested for its ability to react with anti-calmodulin serum. The antiserum bound to ^{125}I-labeled peptide T-1B in direct radioimmunoassay. In addition, unlabeled calmodulin and unlabeled peptide T-1B showed competition curves similar to the respective curves obtained using ^{125}I-labeled calmodulin. The experiments using ^{125}I-labeled peptide T-1B provide direct evidence that the antiserum binds to a distinct fragment of the calmodulin molecule. These data and the quantitative fragmentation experiments demonstrate that the majority, if not all, of the immunoreactivity is contained in peptide T-1B.

DISCUSSION

Two major conclusions can be made from the studies summarized in this report. First, a reproducible and rapid method for the production of anti-calmodulin serum has been described. Second, the majority of the antisera produced by this protocol react with a unique site in the COOH-terminal region of the calmodulin molecule. Specifically, the quantitative immunoreactivity of tryptic peptide T-1B (residues 127–148) and cyanogen bromide peptide CNBr-3B1 (residues 125–144) suggests that the immunoreactive site is contained within the overlapping portion of these peptides, *i.e.* residues 127–144. Reactivity with peptide T-1B has been directly demonstrated by the immunoprecipitation of iodinated peptide T-1B in radioimmunoassay. Consistent with the above data is the

observation that these antisera are nonprecipitating by immunodiffusion criteria. Of the 11 rabbits used in this study, 8 produced antisera that recognized this immunoreactive region. Preliminary results indicate that the exact immunization protocol, the animal species, and the amount of calmodulin administered per injection are not critical for an immune response; however, use of performic acid-oxidized calmodulin is necessary for a reproducible and significant antigenic response.[3]

We do not know how performic acid oxidation renders calmodulin more antigenic. The only detectable chemical change upon oxidation of calmodulin with performic acid is the conversion of the methionyl residues to methionine sulfone. Regardless of the reason, it should be emphasized that the antisera produced by injection of oxidized calmodulin react quantitatively with unoxidized calmodulin. Unoxidized calmodulin is routinely used as the iodinated standard in competition radioimmunoassays, and the competition curve for oxidized calmodulin is identical with that for unoxidized calmodulin. The indistinguishable reactivity of oxidized and unoxidized calmodulin is consistent with other data presented in this report. For example, the observation that the cyanogen bromide digest of calmodulin and purified CNBr-3B1 quantitatively react with the antisera demonstrates that intact methionyl residues are not required for immunoreactivity. In addition, spectroscopic and chemical modification data (for a review, see Ref. 4) suggest that several of the amino acid residues in the proposed immunoreactive domain (residues 127–144) are exposed to the solvent environment in the intact protein.

The destruction of immunoreactivity by digestion of peptide T-1B with chymotrypsin demonstrates that immunoreactivity is not due to a single amino acid residue and suggests the importance of sequences around certain hydrophobic residues, such as Tyr-138 or Phe-141. However, it is clear that iodination of Tyr-138 does not destroy immunoreactivity, since ^{125}I-labeled peptide T-1B is quantitatively immunoprecipitated by anti-calmodulin serum. The direct demonstration of which residues and atoms in the immunoreactive site are necessary for full immunoreactivity can be most readily accomplished by the synthesis of peptide analogs of this region of calmodulin. Such investigations are in progress. Preliminary studies using synthetic segments of the immunoreactive domain have confirmed the conclusions of this study and indicate that the immunoreactive site is smaller than 18 residues in length.[4]

During the course of these studies, other reports (25, 26) concerning production of anti-calmodulin sera and radioimmunoassays for calmodulin have appeared. One method (25) requires the use of dinitrophenylated calmodulin as antigen. The antibodies produced react with dinitrophenylated calmodulin and, to a much lesser extent, with native calmodulin. The other procedure (26) uses native calmodulin as antigen, but requires the use of immunoglobulin fractions purified by calmodulin-Sepharose chromatography in order to detect an immune response. Neither report gives any data on the reproducibility of the injection protocols or the molecular specificity of the antibodies produced. Therefore, it is difficult to directly compare antisera produced by these methods with antisera produced as described in this paper. As shown in Fig. 1 (schedule C), we have confirmed the observation that injection of native calmodulin will lead to the production of anti-calmodulin serum; however, the response is variable and the antiserum titers are low. Similar variable results have been obtained with dinitrophenylated calmodulin. The protocol described in this report is rapid and yields antisera that react with native calmodulin, are of sufficient titer to use dilutions of whole sera in radioimmunoassay, and are truly monospecific.

Because calmodulin is a member of a class of structurally and functionally related proteins, the importance of a reproducible protocol for antibody production and a quantitative analysis of the molecular specificity of the antiserum cannot be overemphasized. Since only a small portion of calmodulin is required for immunoreactivity, it is possible that other structurally and functionally related proteins will show antigenic reactivity. For example, we have shown (27) that a *Chlamydomonas* flagellar protein which may not be a calmodulin will react with anti-calmodulin antiserum. Therefore, caution is necessary in using antisera as specific probes for calmodulin. However, immunoassays can be used in conjunction with enzymatic assays (28), diagnostic gel electrophoresis (12, 27), and structural studies (6, 12, 18, 27) as criteria for the presence of calmodulin. Thus, the ability to produce rapidly and consistently anti-calmodulin serum of useful titer and of defined molecular specificity will allow a clearer interpretation of studies on the role of calmodulin in cell function.

Note Added in Proof—Since submission of this manuscript, we have shown that the reactivity of the antisera with troponin C preparations is probably due to calmodulin contamination. Troponin C preparations that were free of detectable calmodulin contamination did not react when tested up to a 2,000-fold molar excess over that amount of calmodulin required for 50% competition.

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