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Biochemistry

Engineering of site-directed antisera against vertebrate calmodulin by using synthetic peptide immunogens containing an immunoreactive site

(calcium-binding proteins/peptide modeling/anti-epitope/radioimmunoassay/solid-phase peptide synthesis)

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ABSTRACT **Site-directed antisera against vertebrate calmodulin were elicited in rabbits by injection of a synthetic immunogen containing the pentadecapeptide Gly-Gln-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-Thr-Ala-Lys-OH, which corresponds to residues 134–148 of vertebrate calmodulin. A major immunoreactive region (residues 127–144) of calmodulin is found in the COOH-terminal structural domain and an immunoreactive site for one antiserum is contained in the heptapeptide Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2, which corresponds to residues 137–143 of vertebrate calmodulin. This immunoreactive heptapeptide was conjugated to a carrier protein by adding a cysteine residue to the NH_2 terminus of the peptide and coupling the Cys-heptapeptide to the carrier through the thiol group of the cysteine residue. Injection of this Cys-heptapeptide-protein conjugate into rabbits yielded antisera that react with the heptapeptide but not with native calmodulin. Thus, the immunoreactive heptapeptide that is exposed on the surface of calmodulin is immunogenic, but it is not sufficient to elicit antibodies that react with native calmodulin. However, when the Cys-pentadecapeptide corresponding to residues 134–148 and containing the immunoreactive heptapeptide sequence was conjugated to a carrier protein and injected into rabbits, antisera were elicited that react with the intact calmodulin molecule. The affinities and specificities of these antisera for calmodulin are similar to those of antisera elicited by injection of the intact protein and are sufficient for their use in radioimmunoassays. These results indicate that the successful engineering of site-directed antisera against proteins by using synthetic peptide immunogens may require an appropriate intramolecular environment that allows the peptide region to closely approximate the spatial orientation it adopts in the intact protein.**

Vertebrate calmodulin is a 148-residue calcium-binding protein that is highly conserved in structure and function (1–3). A limited number of studies (4–9) indicate that calmodulin structure may be identical among mammals. Calmodulin appears to be highly conserved among vertebrates (4–10), invertebrates (11–13), protozoa (14), and higher plants (2, 3), although isotypes have been reported (15) for sea urchin eggs. In addition to its having been found to have a highly conserved structure and function, calmodulin or calmodulin-like activity has been detected in almost all eukaryotic cells examined, and purified calmodulin has been shown to stimulate a variety of partially purified and purified enzymes in a calcium-dependent manner. Although the physiological significance of most of these stimulatory activities has not been demonstrated, an accumulating body of evidence indicates that calmodulin may be fundamentally important for eukaryotic cell function.

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Immunochemical methods have been especially useful in attempts to define the exact roles of calmodulin in cell function and how these functions might be perturbed in pathophysiological states. In this regard, we have previously reported (16) a reproducible and rapid procedure for producing antisera against vertebrate calmodulin and have developed calmodulin radioimmunoassays using these sera, or affinity-purified IgG fractions, and a native calmodulin standard. We showed that oxidation of methionine residues in calmodulin significantly enhances its immunogenicity in rabbits. The antibodies produced in response to injection of oxidized calmodulin react with native calmodulin, some with apparent association constants of 10^8 to 10^9 M^{-1} for native calmodulin. A major immunoreactive region (residues 127–144) for these antisera is found in the COOH-terminal domain of calmodulin, and an immunoreactive site for the antisera from one rabbit was shown (17) to be contained in a heptapeptide corresponding to residues 137–143 of vertebrate calmodulin.

These site-specific antisera have been important tools in biological studies and in attempts to correlate structural domains with functional domains by using immunochemical mapping approaches. The antisera specific for residues 137–143 appear to bind to monomeric calmodulin as well as to calmodulin in supramolecular complexes such as muscle phosphorylase kinase, exhibit differential reactivity with calmodulin isotypes from sea urchin eggs, and react poorly with plant or protozoan calmodulins (3). Among calmodulins, the only known amino acid sequence difference in the 137–143 region is at position 143, where glutamine is found in vertebrate calmodulin and an arginine or lysine is found in invertebrate, plant, or protozoan calmodulins. Thus, the ability to reproducibly elicit site-directed antisera that would react with this region of the calmodulin molecule would be useful in both immunochemical mapping studies and studies that attempt to differentially detect and measure calmodulin isotypes or calmodulin and closely related proteins in the same sample.

To develop procedures for engineering antisera against specific regions of calmodulin and related proteins, we synthesized peptide segments that contain the heptapeptide immunoreactive site from vertebrate calmodulin, conjugated them to carriers, and injected them into rabbits. We report here the results of these attempts to generate reproducibly site-specific antisera.

MATERIALS AND METHODS

Materials. Chemicals were purchased as follows: N^{α}-*tert*-butoxycarbonyl (Boc) derivatives of *S*-(4-methoxybenzyl)-L-cys-

Abbreviation: KLH, keyhole limpet hemocyanin.

Table 1. Immunoreactivity of synthetic peptides with antisera from rabbit 654

Peptide code	Peptide structure*	Mean dose for 50% competition with calmodulin, pmol†	Range of relative immunoreactivity, %‡
Chicken gizzard calmodulin (1–148)		3.0 ± 1.4	100
14	H-Cys-Gly-Gln-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-Thr-Ala-Lys-OH (134 137 140 143 145 148)	0.6 ± 0.1	429–600
1	H-Gly-Gln-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-NH_2	2.4 ± 1.1	86–231
2	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-NH_2	3.8 ± 2.0	52–167
3	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-NH_2	5.1 ± 3.0	38–143
4	H-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-NH_2	10 ± 5	20–60
5	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	32 ± 15	6–18
6	H-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-NH_2	36 ± 22	5–21
7	H-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	32 ± 11	9–14
8	H-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-NH_2	400	0.75
9	H-Tyr-Glu-Glu-Phe-Val-Gln-Met-NH_2	950	0.32
10	H-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	1,850	0.16
11	H-Tyr-Glu-Glu-Phe-Val-NH_2	>10,000§	<0.03
12	H-Glu-Glu-Phe-Val-Gln-Met-Met-NH_2	33,000	0.01

* Peptide sequences are based on the amino acid sequence of bovine brain calmodulin (3, 4).
† Values for peptides 1–7 and 14 represent the mean ± SD of three to eight separate assays. Values for peptides 8–12 represent the mean of duplicate determinations from a single assay in which SD was less than 7%.
‡ Range of molar immunoreactivities of the synthetic peptides relative to the mean value obtained with chicken gizzard calmodulin.
§ The highest dose tested (10,000 pmol) did not give 50% competition.

teine, *O*-(2,6-dichlorobenzyloxycarbonyl)-L-lysine, and other amino acids from Peninsula Laboratories (San Carlos, CA); *N,N'*-dicyclohexylcarbodiimide from Pierce; chicken ovalbumin from Millipore; *N*-succinimidyl 3-(2-pyridyldithio)propionate from Pharmacia; keyhole limpet hemocyanin (KLH) from Calbiochem; normal rabbit serum and goat anti-rabbit serum from Cappel Laboratories (Cochranville, PA); sodium [^{125}I]iodide (carrier-free, 100 mCi/ml; 1 Ci = 3.7 × 10^{10} Bq) from Amersham. Female New Zealand White rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN). Dialysis tubing was Spectrapor grade (American Scientific Products, Stone Mountain, GA) with a nominal molecular weight cutoff of 12,000–14,000.

Peptide Synthesis. Peptide amides **1–12** (Table 1) and the octapeptide amide **13** (Cys-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2) were synthesized by the solid-phase method (18, 19). Briefly, the desired protected peptides were assembled on α-aminobenzyl-poly(styrene/1% divinylbenzene) beads (Beckman), cleaved from the beads and deprotected by treatment with 9:1 (vol/vol) liquid HF/4-cresol, and purified by reverse-phase liquid chromatography on octadecylsilica. The hexadecapeptide acid **14** (Table 1) was prepared by stepwise addition of Boc-amino acids to N^{α}-Boc-N^{ϵ}-(2-chlorobenzyloxycarbonyl)-L-lysine esterified to 4-hydroxyphenylacetamidomethyl-poly(styrene/1% divinylbenzene) beads (20) at a loading of 0.32 mmol of lysine per gram of polystyrene. Each peptide was homogeneous by re-

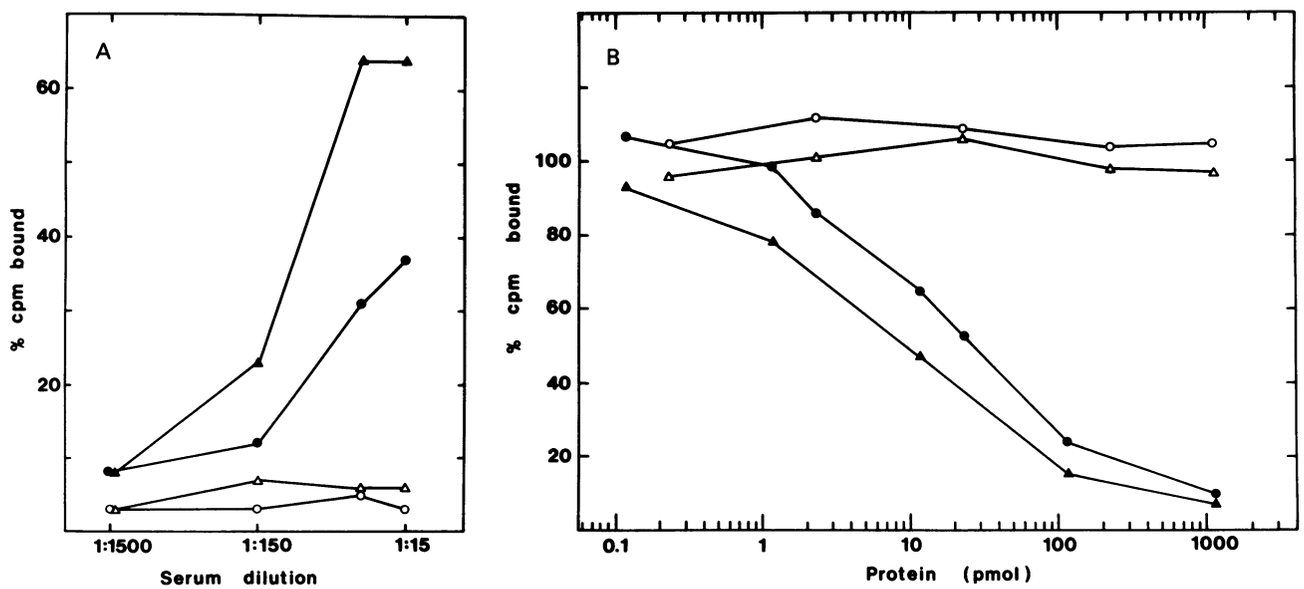

FIG. 1. Characterization by radioimmunoassay of antisera against the octapeptide **13**-KLH conjugate. (*A*) Direct radioimmunoassay of binding of antisera from rabbits 77 (▲, △) and 78 (●, ○) to ^{125}I-labeled peptide **7** (●, ▲) or ^{125}I-labeled calmodulin (○, △). Various dilutions of antiserum were added to a fixed concentration of ^{125}I-labeled peptide **7** (73,000 cpm; ≈0.1 pmol) or ^{125}I-labeled calmodulin (70,000 cpm; ≈0.07 pmol) and incubated and processed as described (16). The ordinate represents the percentage of the radioactivity bound. (*B*) Reactivity of antisera from rabbits 77 (▲, △) and 78 (●, ○) in competition radioimmunoassay using ^{125}I-labeled peptide **7** as tracer. Various concentrations of unlabeled peptide **7** (●, ▲) and calmodulin (○, △) were mixed with antiserum (1:75 final dilution) and incubated overnight at 4°C. A fixed concentration of ^{125}I-labeled peptide **7** (50,000 cpm; ≈0.08 pmol) was added and the mixture was processed as described (16). The degree of competition is expressed as a percentage of the radioactivity bound in the absence of competing peptide or protein.

verse-phase chromatography and thin-layer chromatography and gave the expected amino acid ratios (data not shown).

Preparation of Peptide-Protein Conjugates. Peptide 13 was coupled to KLH by the iminothiolane procedure (21). Peptide 14 was coupled to ovalbumin by using *N*-succinimidyl 3-(2-pyridyldithio)propionate essentially as described (22). Briefly, after desalting on Sephadex G-25, 6.5 mg of ovalbumin in 0.8 ml of 0.01 M sodium phosphate, pH 7.5, was mixed with 1.0 mg of coupling agent in 0.2 ml of ethanol for 5 min. The reaction mixture was immediately passed through a column (0.9 × 25 cm) of Sephadex G-25 equilibrated in 0.01 M sodium phosphate, pH 7.5. The fraction containing the activated ovalbumin (4 thiopyridine groups per molecule of ovalbumin) was mixed with 1.5 eq of peptide 14 in 1.0 ml of 0.01 M sodium phosphate, pH 7.5, for 24 hr at 25°C. Unreacted peptide 14 and crosslinking reagent were removed by dialysis. Approximately 20 copies of octapeptide 13 were conjugated to each molecule of KLH and 3.6 copies of hexadecapeptide 14 were conjugated to each molecule of ovalbumin.

Injection Schedule. Two different peptide-protein conjugates, peptide 13-KLH and peptide 14-ovalbumin, were injected subcutaneously. The immunogens were emulsified in

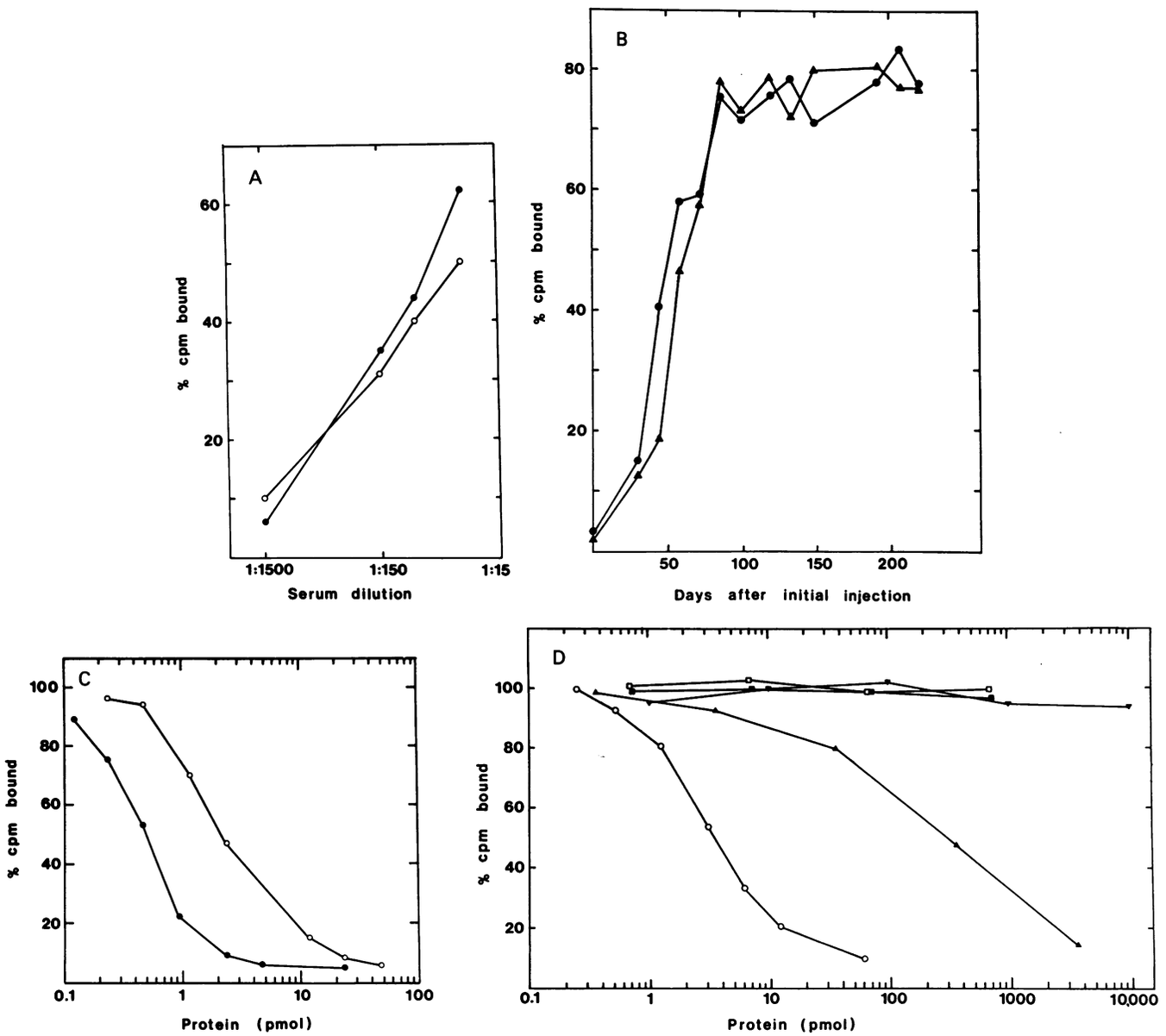

FIG. 2. Characterization by radioimmunoassay of antisera against the hexadecapeptide 14-ovalbumin conjugate. (*A*) Direct radioimmunoassay of binding of antiserum from rabbit 653 to ^{125}I-labeled peptide 14 (●) or ^{125}I-labeled calmodulin (○). The radioimmunoassay was done as described in the legend of Fig. 1 except that ^{125}I-labeled peptide 14 (38,000 cpm; ≈0.6 pmol) and ^{125}I-labeled calmodulin (45,000 cpm; ≈0.05 pmol) were used. (*B*) Time course of antibody production. Rabbits 653 (▲) and 654 (●) were injected with peptide 14-ovalbumin as described in *Materials and Methods*. Reactivity of serum from each bleeding was analyzed by direct radioimmunoassay using fixed concentrations of antisera (1:30 final dilution) and ^{125}I-labeled calmodulin (61,000 cpm; ≈0.03 pmol). (*C*) Reactivity in competition radioimmunoassay of antiserum from rabbit 654. Various concentrations of chicken gizzard calmodulin (○) and peptide 14 (●) were mixed with antiserum (1:150 final dilution) and incubated for 7 hr at 4°C. A fixed concentration of ^{125}I-labeled calmodulin (55,000 cpm; ≈0.03 pmol) was added and the mixture was processed as described in the legend of Fig. 1. (*D*) Specificity of the immunoreactivity of antiserum from rabbit 654. Competition radioimmunoassay was done as described in the legend of Fig. 1, using antiserum (1:300 final dilution), ^{125}I-labeled calmodulin (67,000 cpm; ≈0.05 pmol), and various concentrations of the following competing proteins: chicken gizzard calmodulin (○), spinach calmodulin (▲), rabbit skeletal muscle troponin C (▼), bovine brain S100α (■), and bovine brain S100β (□).

either complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (subsequent injections). Two rabbits (nos. 77 and 78) were injected with peptide **13**-KLH (150 nmol of peptide **13** per rabbit per injection) on days 0, 6, 8, 11, 13, and 15, and were bled on days 22, 36, and 51. Two rabbits (nos. 653 and 654) were injected with peptide **14**-ovalbumin (50–60 nmol of peptide **14** per rabbit per injection) on days 0, 14, 16, 18, 21, and 23, and were bled on day 30. Rabbits 653 and 654 were boosted approximately every 2 weeks and bled 7–10 days after each boost. Serum was obtained as described (16).

Other Methods. Calmodulin (16), rabbit skeletal muscle troponin C (23), and bovine brain proteins S100α and S100β (24) were prepared as previously described. Iodinations and radioimmunoassays were done as described (16). Protein concentrations were determined by amino acid analysis (25).

RESULTS

To determine whether the immunoreactive site heptapeptide **7** (see Table 1) could also be immunogenic, we prepared synthetic octapeptide **13** consisting of the immunoreactive heptapeptide **7** plus an NH_2-terminal cysteine residue and coupled the octapeptide to a carrier molecule. Fig. 1 shows the immune responses of two rabbits injected with the peptide **13**-KLH conjugate. The sera bound to iodinated heptapeptide **7** but not to iodinated calmodulin. In competition radioimmunoassay (Fig. 1*B*), unlabeled heptapeptide **7** competed with the iodinated heptapeptide for binding to the antisera, whereas vertebrate calmodulin did not compete even at the highest level tested (1,000 pmol). These data demonstrate that the conjugated octapeptide containing the immunoreactive heptapeptide **7** is immunogenic but that the antisera recognize the peptide and not the native protein containing the same amino acid sequence.

Our goal was to elicit antisera that would react with the native protein. Therefore, we prepared synthetic hexadecapeptide **14** containing residues 134–148 of vertebrate calmodulin plus an NH_2-terminal cysteine residue and coupled it to ovalbumin. The antisera elicited by injection of the peptide **14**-ovalbumin conjugate were immunoreactive with both iodinated peptide **14** and iodinated calmodulin (Fig. 2*A*). Fig. 2*A* is a representative experiment showing the response of one of the rabbits; the reactivity of the other rabbit was similar. Fig. 2*B* shows the time course of appearance of anti-calmodulin reactivity in the two rabbits. There was no reactivity in either preimmune serum.

The ability of the antisera to bind to iodinated calmodulin allowed the establishment of a radioimmunoassay using ^{125}I-labeled calmodulin as the tracer. A competition radioimmunoassay showing the reactivity of unlabeled calmodulin and the hexadecapeptide **14** is illustrated in Fig. 2*C*. For both rabbit antisera, peptide **14** was more reactive in competition radioimmunoassay than was intact calmodulin. These results are similar to those previously obtained (16, 17) with antiserum prepared against the 148-residue calmodulin molecule, in which synthetic peptides or cleavage peptides competed as well as or better than intact calmodulin.

The specificity of the antisera produced by injection of hexadecapeptide **14** was examined by determining the ability of several structurally and functionally related proteins to compete quantitatively with ^{125}I-labeled calmodulin in the radioimmunoassay. These antisera show no reactivity with troponin C, S100α, or S100β at doses up to 1,000–10,000 pmol (Fig. 2*D*). Spinach calmodulin shows some crossreactivity, but approximately 100 times more protein is required for 50% competition (Fig. 2*D*). This result is not surprising (17), because spinach calmodulin differs from vertebrate calmodulin in three amino acid residues in the 134–148 region (Val/Ile-136, Gln/Lys-143, and Met/Val-144).

In order to examine the residues within the hexadecapeptide that are required for immunoreactivity, a series of synthetic peptides was examined by competition radioimmunoassay. Fig. 3 shows competition curves for representative peptides and Table 1 summarizes the immunoreactivity of the synthetic peptides with antiserum from one of the rabbits injected with the hexadecapeptide **14**-ovalbumin conjugate (rabbit 654). The peptides can be placed in three groups on the basis of their immunoreactivity. First, peptides **1–3** and **14** show reactivity comparable to or better than that of intact calmodulin and are represented in Fig. 3 by decapeptide **2**. Second, peptides **4–7** are 1/3rd to 1/12th as reactive as intact calmodulin and are represented by heptapeptide **7**. Third, peptides **8–12** show little or no reactivity and are represented by octapeptide **8**. Our data demonstrate that amino acid residues Gly-134, Gln-135, Thr-146, Ala-147, and Lys-148 present in the immunogenic peptide **14** are not required for quantitative immunoreactivity with antiserum 654.

Radioimmunoassays using antisera from the other rabbit injected with the hexadecapeptide (rabbit 653) gave results qualitatively similar (Fig. 2*B*) to those using antisera from rabbit 654. However, the residues within the hexadecapeptide that are required for immunoreactivity differ for the two rabbits. As described above, peptide **2** is as immunoreactive and peptide **7** is only 1/10th as reactive as intact calmodulin when antisera from rabbit 654 are used. In contrast, when antisera from rabbit 653 were tested, peptide **2** was 1/150th as reactive as cal-

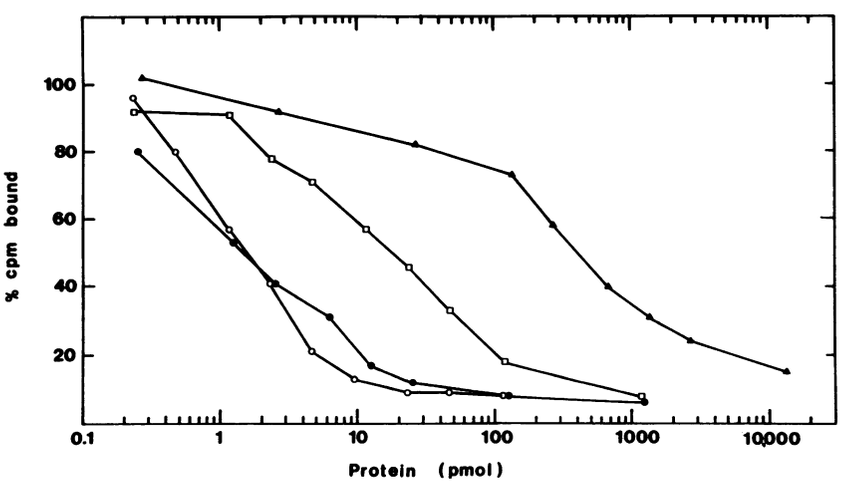

FIG. 3. Immunoreactivity of synthetic peptides with antiserum from rabbit 654. Competition radioimmunoassay was done as described in the legend of Fig. 1, using antiserum (1:255 final dilution), ^{125}I-labeled calmodulin (62,000 cpm; ≈0.04 pmol), and various concentrations of the following competing protein and peptides: chicken gizzard calmodulin (○), decapeptide **2** (●), heptapeptide **7** (□), and octapeptide **8** (▲).

modulin and peptide **7** showed no reactivity at the highest dose tested (12,000 pmol).

DISCUSSION

In this study we have (*i*) demonstrated the feasibility of using synthetic peptide immunogens to elicit site-directed antisera that react with native calmodulin, (*ii*) shown that the immunoreactive heptapeptide **7** (residues 137–143 of vertebrate calmodulin) is also an immunogenic region, capable of eliciting an immune response in rabbits, and (*iii*) demonstrated that the production of antisera against an intact protein by using a surface-exposed segment is not as simple as previously assumed because it may require presentation of the peptide segment in an orientation similar to that in the intact protein.

The ability to use synthetic peptide immunogens to produce antibodies of defined specificity is an important advance in immunochemistry. The design of the synthetic immunogen is obviously one of the most important considerations in attempts to elicit antibodies that react with the native protein. Previously (26), essentially any peptide that was at least seven amino acid residues long and exposed on the surface of a protein molecule appeared to be capable of serving as a peptide immunogen for eliciting antibodies that react with an intact protein molecule. We have demonstrated here that for vertebrate calmodulin these criteria are not sufficient. Heptapeptide **7**, which is an immunoreactive region and is exposed on the surface in the vertebrate calmodulin molecule, is not sufficient to elicit antibodies that react with intact calmodulin.

When the immunoreactive heptapeptide **7** was presented within a larger peptide (hexadecapeptide **14**) in a molecular orientation presumably more like that in the native calmodulin molecule, antibodies were elicited that react with intact calmodulin. These antibodies show apparent association constants on the order of 10^8 to 10^9 M^{-1}, values comparable to those of some anti-calmodulin antibodies prepared against the intact protein (16). The affinity and specificity of these anti-peptide antisera were sufficient to allow development of radioimmunoassays using these sera, iodinated vertebrate calmodulin as tracer, and intact calmodulin as standard. The affinity and specificity of these antibodies also allow their potential use as immunoadsorbents to purify calmodulin as a component of supramolecular complexes. Finally, the ability to design antibodies against selected regions of the calmodulin molecule will be useful in structure–function studies to provide specific molecular probes for the immunochemical mapping of functional domains on calmodulin.

In a more general sense, the studies reported here provide a precedent for a rational approach to the development of specific immunochemical assays for calcium-modulated proteins that avoids and circumvents the limitations inherent in "shotgun" approaches. For example, we have previously demonstrated (16) that most of the antibodies elicited by injection of vertebrate calmodulin react with a major immunoreactive region in the COOH-terminal domain of calmodulin. Most of the amino acid sequence differences among calmodulins from vertebrates, invertebrates, protozoa, and plants are found in the COOH-terminal one-third of the molecule. The approaches described here may allow the engineering of antisera that react with other regions of calmodulin, such as the highly conserved second domain. Whereas hybridoma technology may yield abundant supplies of antibodies with defined chemical specificities, antibodies from hybridomas that are readily generated may not distinguish highly related members of a protein superfamily. For example, when purified S100, a member of the calcium-modulated protein family, was used as an immunogen, the resultant monoclonal antibodies reacted with multiple S100 proteins (27). In addition, the desired hybridomas may be produced in such low abundance that their detection is difficult. On the basis of our results with calmodulin, it is now feasible to attempt production of specific, site-directed antisera against the various proteins in the calmodulin superfamily of calcium-binding proteins. Clearly, in some cases appropriately designed synthetic immunogens eliminate the necessity of using hybridoma technology.

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1. Klee, C. B. & Vanaman, T. C. (1982) *Adv. Protein Chem.* **35**, 213–321.
2. Burgess, W. H., Schleicher, M., Van Eldik, L. J. & Watterson, D. M. (1983) in *Calcium and Cell Function*, ed. Cheung, W. Y. (Academic, New York), pp. 209–261.
3. Watterson, D. M., Burgess, W. H., Lukas, T. J., Iverson, D., Marshak, D. R., Schleicher, M., Erickson, B. W., Fok, K.-F. & Van Eldik, L. J. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 205–226.
4. Watterson, D. M., Sharief, F. & Vanaman, T. C. (1980) *J. Biol. Chem.* **255**, 962–975.
5. Kasai, H., Kato, Y., Isobe, T., Kawasaki, H. & Okuyama, T. (1980) *Biomed. Res.* **1**, 248–264.
6. Grand, R. J. A. & Perry, S. V. (1978) *FEBS Lett.* **92**, 137–142.
7. Dedman, J. R., Jackson, R. L., Schreiber, W. E. & Means, A. R. (1978) *J. Biol. Chem.* **253**, 343–346.
8. Grand, R. J. A., Schenolikar, S. & Cohen, P. (1981) *Eur. J. Biochem.* **113**, 359–367.
9. Sasagawa, T., Ericsson, L. H., Walsh, K. A., Schreiber, W. E., Fischer, E. H. & Titani, K. (1982) *Biochemistry* **21**, 2565–2569.
10. Lagace, L., Chandra, T., Woo, S. L. C. & Means, A. R. (1983) *J. Biol. Chem.* **258**, 1684–1688.
11. Jamieson, G. A., Jr., Hayes, A., Blum, J. J. & Vanaman, T. C. (1980) in *Calcium Binding Proteins: Structure and Function*, eds. Siegel, F. L., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Wasserman, R. H. (Elsevier/North-Holland, New York), pp. 165–172.
12. Takagi, T., Nemoto, T., Konishi, K., Yasawa, M. & Yagi, K. (1980) *Biochem. Biophys. Res. Commun.* **96**, 377–381.
13. Toda, H., Yazawa, M., Kondo, K., Honma, T., Narita, K. & Yagi, K. (1981) *J. Biochem.* **90**, 1493–1505.
14. Yazawa, M., Yagi, K., Toda, H., Kondo, K., Narita, K., Yamazaki, R., Sobue, K., Kakiuchi, S., Nagao, S. & Nozawa, Y. (1981) *Biochem. Biophys. Res. Commun.* **99**, 1051–1057.
15. Burgess, W. H. (1982) *J. Biol. Chem.* **257**, 1800–1804.
16. Van Eldik, L. J. & Watterson, D. M. (1981) *J. Biol. Chem.* **256**, 4205–4210.
17. Van Eldik, L. J., Watterson, D. M., Fok, K.-F. & Erickson, B. W. (1983) *Arch. Biochem. Biophys.* **227**, 522–533.
18. Erickson, B. W. & Merrifield, R. B. (1976) in *The Proteins*, eds. Neurath, H. & Hill, R. L. (Academic, New York), 3rd Ed., Vol. 2, pp. 255–527.
19. Ohga, K., Incefy, G. S., Fok, K.-F., Erickson, B. W. & Good, R. A. (1983) *J. Immunol. Methods* **57**, 171–184.
20. Mitchell, A. R., Erickson, B. W., Ryabtev, M. N., Hodges, R. S. & Merrifield, R. B. (1976) *J. Am. Chem. Soc.* **98**, 7357–7362.
21. King, T. P., Li, Y. & Kochoumian, L. (1978) *Biochemistry* **17**, 1499–1506.
22. Carlsson, J., Drevin, H. & Axen, R. (1977) *Biochem. J.* **173**, 723–737.
23. Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743.
24. Marshak, D. R., Watterson, D. M. & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6793–6797.
25. Van Eldik, L. J. & Watterson, D. M. (1979) *J. Biol. Chem.* **254**, 10250–10255.
26. Lerner, R. A. (1982) *Nature (London)* **299**, 592–596.
27. Haan, E. A., Boss, B. D. & Cowan, W. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7585–7589.