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# Peptide antisera as sequence-specific probes of protein conformational transitions: Calmodulin exhibits calcium-dependent changes in antigenicity

(protein folding/synthetic peptides)

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ABSTRACT **Local changes in conformation between the calcium-saturated and calcium-free forms of calmodulin were monitored using antisera to four peptides corresponding to three helical regions of the calcium-saturated protein. The N-terminal helix was monitored using antiserum to residues 9–19, calmodulin-(9–19); the C-terminal helix using antiserum to residues 141–148, calmodulin-(141–148); and the long central helix with antisera to residues 68–79 and 80–92, calmodulin-(68–79) and -(80–92). Crossreactivities of peptide antisera with calmodulin (either in the presence or absence of calcium) were determined using solution-phase and solid-phase immunoassays. When examined by the fluid-phase assay, all four peptides elicited antibody that precipitated radiolabeled apocalmodulin but not the calcium-saturated form of the protein. Similarly, when calmodulin was immobilized on a solid-support, only the calcium-free form readily bound the antibodies to calmodulin-(80–92) and -(141–148). In addition, the crossreactivity of antiserum to calmodulin-(68–79) with calcium-saturated calmodulin in solid phase was reduced by ≈40% relative to reactivity with apocalmodulin. According to the x-ray crystal structure of $Ca^{2+}$-saturated calmodulin and the antigenic reactivity of calmodulin for the peptide antisera in the absence of calcium, the regions of the protein monitored by these antisera are exposed to the surface in both conformational states and probably accessible to specific antibodies. The apparent preference of peptide antibodies for one conformation of the molecule suggests that changes in the conformation of calmodulin occur in cognate sequences that are transformed by calcium from antigenic, flexible structures to less antigenic, relatively helical structures. Peptide antibodies may be employed as sequence-specific reporter molecules to monitor local conformational changes providing the cognate sequence is sterically accessible to antibody in both states but antigenic in only one.**

Antibodies to synthetic peptides corresponding to linear segments of a protein will frequently react with the homologous sequence in the native folded protein (cognate sequence) provided the region is exposed to the surface and exhibits a repertoire of conformations similar to the immunizing peptide (1). Peptide antibodies react strongly with highly mobile regions of myohaemerythrin but not with well-ordered regions of the same protein (2). Similarly, the location of the linear antigenic determinants of tobacco mosaic virus protein, lysozyme, and myoglobin has been correlated with regions of high segmental mobility as determined by x-ray crystallographic temperature factors (3). An additional characteristic of antigenic regions in proteins is their surface accessibility to the antigen combining site of specific antibody (4, 5). These findings and others (6) suggest that peptide antibodies might be used to detect changes in the antigenicity of the cognate sequence indicative of local changes in segmental mobility and solvent accessibility for proteins that undergo conformational shifts. Loss of antigenicity may indicate either that the region has become sterically inaccessible to the peptide antibody or that it has adopted a significantly different or more constrained conformation.

In this report, the use of peptide antibodies to monitor sequence-specific conformational changes has been examined with synthetic peptides corresponding to regions of calmodulin, a protein that adopts at least two defined solution geometries depending on the cytosolic calcium concentration—($[Ca^{2+}] < 10^{-7}$ M) or ($[Ca^{2+}] > 10^{-5}$ M). In the presence of $Ca^{2+}$, the $\alpha$-helical content of calmodulin is estimated by circular dichroism and optical rotatory dispersion to increase by 5–15% to 40–60% (7, 8), and the molecule is transformed into a multienzyme activator (9, 10). X-ray crystallographic studies of calcium-saturated calmodulin have revealed a dumbbell-like structure in which a long central $\alpha$-helix divides the molecule into two regions, each of which contain a pair of homologous $Ca^{2+}$ binding domains and three small $\alpha$-helices (11). We have prepared antibodies to four synthetic peptides corresponding to calmodulin residues 9–19, 68–79, 80–92, and 141–148. [Hereafter these peptides will be referred to as calmodulin-(9–19), -(68–79), -(80–92), and -(141–148), respectively.] These peptides span three of the seven regions of calmodulin identified as being helical in the $Ca^{2+}$-saturated conformer. These antisera were used to monitor local changes in the conformation of calmodulin in the presence and absence of $Ca^{2+}$.

## EXPERIMENTAL PROCEDURES

**Materials.** Proteins and chemicals were obtained from the following vendors: bovine brain calmodulin from Calbiochem-Behring; phenyl-Sepharose CL-4B, bovine serum albumin (BSA), and bovine thyroglobulin from Sigma; $Na^{125}I$ and *N*-succinimidyl 3-(hydroxy-3,5-[$^{125}$I]diiodophenyl)propionate (Bolton–Hunter reagent) from Amersham; protein A and protein A-Sepharose CL-4B from Pharmacia.

**Preparation of Peptides and Peptide Antisera.** The peptides denoted in Fig. 1 were prepared by Merrifield solid-phase synthesis (12) and partially purified by reverse-phase chromatography as described (13). The composition and concentration of each synthetic peptide was determined by amino acid analysis. Peptides were synthesized with a N- or C-terminal nonnatural cysteine through which they were

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Abbreviation: BSA, bovine serum albumin.

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coupled in one orientation to thyroglobulin. Preparation of these conjugates has been described (14). One to three New Zealand White rabbits were immunized intramuscularly and subscapularly with 1 mg of conjugate emulsified in complete Freund's adjuvant. Eight weeks later, 0.5 mg of the same conjugate in incomplete Freund's adjuvant was administered. Sera were harvested 9 days later.

**Solid-Phase Immunoassays.** The wells of polyvinyl chloride plates were exposed overnight to unconjugated synthetic peptide (10–60 μg per well), BSA (10 μg per well), or calmodulin (10 μg per well) dissolved in 0.02 M Tris·HCl, pH 7.6, containing 0.1 M NaCl, 0.2% BSA, and 1 mM $CaCl_2$ (buffer A) or 1 mM EGTA (buffer B). Immune serum was diluted from 1:100 to 1:50,000 in either buffer A or buffer B containing Brij 35 (0.1%), the dilutions were added to each well, and the reactants were incubated for 2 hr at room temperature. The wells were then washed with buffer A or buffer B containing 0.1% Brij 35, and the bound antibody was detected with protein A iodinated by the method of Greenwood *et al.* (15) to a specific activity of 1–2 × $10^3$ Ci/mmol (1 Ci = 37 GBq). Iodinated protein A was diluted in either buffer A or buffer B containing 0.1% Brij 35, and 50,000–80,000 cpm was added to each well. The reaction was allowed to proceed for 1 hr at room temperature, and the wells were washed with buffer A or buffer B containing 0.1% Brij 35. The wells were then cut from the plate, and radioactivity was measured in a γ counter.

**Solution-Phase Immunoassays.** Calmodulin was iodinated by the method described by Bolton and Hunter (16). The iodinated reagent (0.5 mCi supplied in benzene) was evaporated to dryness under a stream of air. Calmodulin (1.25 μg) in 50 μl of 0.1 M sodium borate buffer, pH 8.5, and 2 mM $CaCl_2$ were added to the dried residue and incubated for 30 min at 4°C. The reactants were then diluted into 1 ml of 0.02 M Tris·HCl, pH 7.2, and 0.1 M NaCl (Tris saline buffer) containing 2 mM $CaCl_2$ and BSA at 0.1 mg/ml. Iodinated calmodulin was separated from the unreacted Bolton–Hunter reagent by hydrophobic chromatography on a phenyl-Sepharose column (1 cm × 4 cm) that had been equilibrated with Tris saline buffer containing 2 mM $CaCl_2$. The bound radiolabeled calmodulin was washed with 10 ml of Tris saline buffer in the presence of 2 mM $CaCl_2$ and eluted in Tris saline buffer containing 2 mM EGTA. The final specific activity of the labeled calmodulin was estimated to be 6 × $10^2$ Ci/mmol.

The specificity of the peptide antisera was monitored by immunoprecipitation of iodinated calmodulin. Radiolabeled calmodulin was diluted to $10^6$ cpm/ml in Tris saline buffer containing 0.2% BSA and either 2 mM $CaCl_2$ or 2 mM EGTA. Fifty-microliter aliquots of the diluted labeled protein was dispensed into 1.5-ml microcentrifuge tubes. Antiserum was added to the first tube, and serial 5-fold dilutions were prepared, yielding dilutions ranging from 1:20 to 1:62,500. Antigen–antibody reactions were allowed to proceed in solution for 1 hr at room temperature. Antibody was precipitated from solution by adding 25 μl of a protein A-Sepharose suspension [6.25% (wt/vol) in Tris saline buffer containing 0.2% BSA and either 2 mM $CaCl_2$ or 2 mM EGTA]. Following a 1-hr incubation period at room temperature, the protein A-Sepharose was recovered by centrifugation, and the supernatant was removed by suction through a drawn glass pipet. The pelleted protein A-Sepharose was washed in 1 ml of Tris saline buffer containing 0.2% BSA and either 2 mM $CaCl_2$ or 2 mM EGTA. After centrifugation and removal of the supernatant, radioactivity in the tubes was measured in a γ counter.

## RESULTS

Calmodulin is a 148-residue polypeptide that possesses four homologous calcium binding sites. Binding of calcium to these sites induces a conformational shift that results in an increased α-helical content and confers the capacity to interact with enzymes, peptides, and hydrophobic molecules (9, 10, 17). Detailed structural information about the apoprotein is unavailable. However, the x-ray crystal structure of $Ca^{2+}$-saturated calmodulin at 3-Å resolution has been reported (11). Each calcium binding site is flanked by N- and C-terminal α-helical segments. Calcium binding sites I and II are separated from sites III and IV by a long, solvent-exposed α-helix encompassing residues 65–92 (Fig. 1). The molecule also contains six shorter helices which consist of residues 7–19, 29–39, 46–55, 102–112, 119–128, and 138–148 (Fig. 1); the total helical content of the $Ca^{2+}$-saturated conformer by x-ray crystallography is 63% (11).

In this study four synthetic peptides were prepared: calmodulin-(9–19), -(68–79), -(80–92), and -(140–148) (Fig. 1). Based on the crystallographic data, these regions appear to be α-helical and solvent-exposed in the calcium-saturated molecule. Each peptide was linked to thyroglobulin through a nonnatural N- or C-terminal cysteine. The antibodies elicited by these peptide-carrier conjugates were used as sequence-specific probes to monitor $Ca^{2+}$-induced changes in the antigenicity of calmodulin. Experiments were carried out at millimolar concentrations of $CaCl_2$ or EGTA to ensure that only one conformational state of calmodulin was being monitored. The metal chelator EGTA itself does not affect $Ca^{2+}$ binding to calmodulin (19).

**The Immunogenicities of the Four Peptide-Carrier Conjugates Are Similar.** Reactivities of peptide antisera to the immunizing peptide were examined by a solid-phase immunoassay. Neither the preimmune sera nor protein A bound significantly to wells coated with peptides, BSA, or calmodulin (results not shown). However, antisera elicited by each peptide-carrier conjugate recognized the homologous peptide in roughly equal titers indicating that the immunogenicity of the four peptides was approximately the same (Fig. 2). The antigenicity of the peptides was not affected by the presence of calcium or EGTA, suggesting that

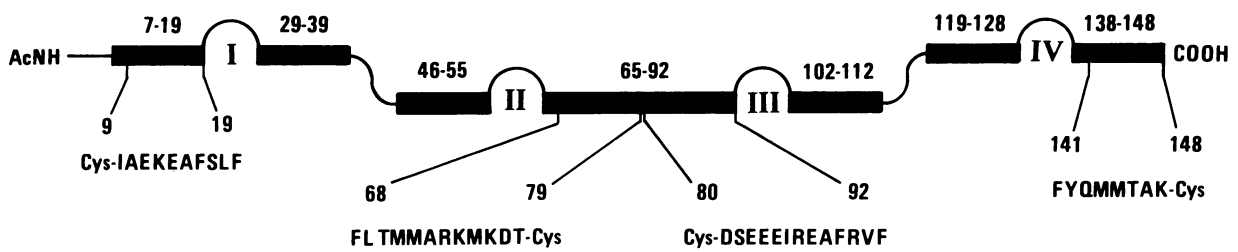


FIG. 1. Schematic representation of bovine brain calmodulin. The closed rectangles depict the α-helical regions flanking each of the four calcium binding loops (roman numerals I–IV). The residue numbers that delimit each of the seven helices of $Ca^{2+}$-saturated calmodulin are indicated above the closed rectangles (11, 18). The four synthetic peptides prepared for this study represent parts of three helical domains of calmodulin. The sequence of each synthetic peptide in a one-letter amino acid code is depicted below the corresponding helix. They were prepared with an additional cysteine either at their N or C terminus for conjugation to a carrier protein (thyroglobulin).

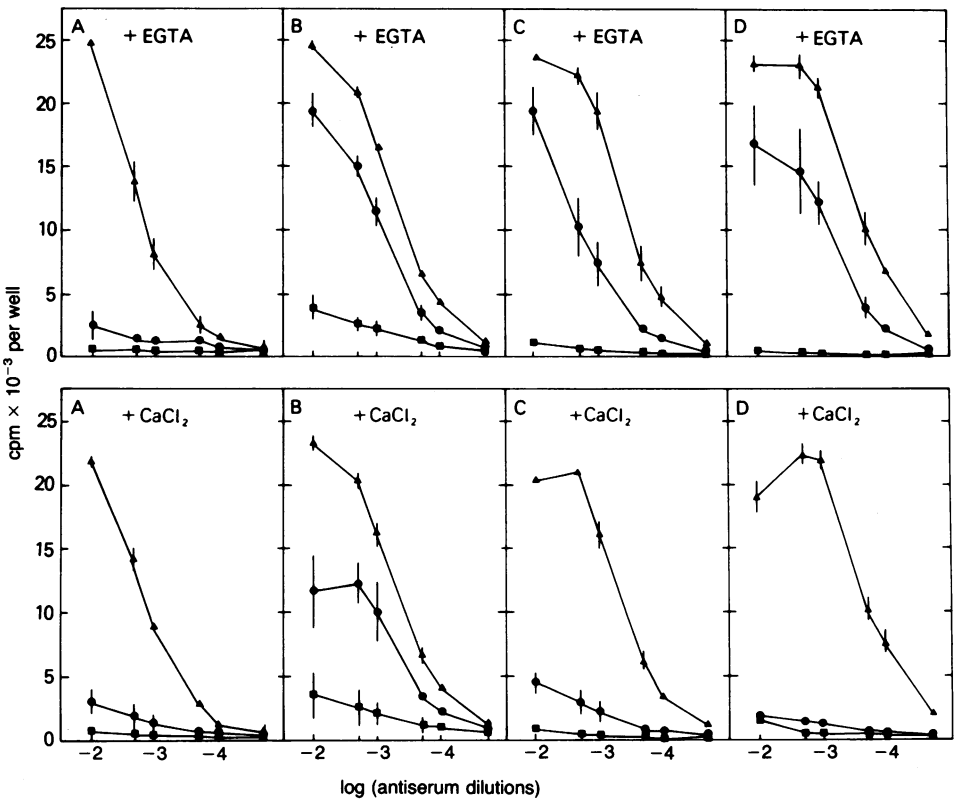


FIG. 2. Effect of EGTA and calcium on the ability of peptide antisera to crossreact with calmodulin bound to a solid support. (*A*s) Calmodulin-(9–19) antiserum. (*B*s) Calmodulin-(68–79) antiserum. (*C*s) Calmodulin-(80–92) antiserum. (*D*s) Calmodulin-(141–148) antiserum. Each was incubated with either BSA (■), bovine brain calmodulin (●), or the homologous peptide immunogen (▲). Each point represents the average (± SD) of assays performed in triplicate. The results depict data obtained with the antiserum from a single immunized rabbit.

$Ca^{2+}$-induced changes in conformation do not occur in calmodulin peptides that lack a $Ca^{2+}$ binding site.

**The Peptide Antisera Detect Three Cognate Sequences of Calmodulin Bound to a Solid Support That Undergo Calcium-Dependent Changes in Antigenicity.** Antiserum elicited by calmodulin-(9–19) did not recognize calmodulin (Fig. 2*A*) in the presence of EGTA or $CaCl_2$, suggesting that the cognate sequence is either sterically inaccessible to specific antibody when calmodulin is bound to a solid support or that it maintains a constrained conformation that is $Ca^{2+}$ independent and does not mimic the conformational flexibility of the immunizing peptide. Antiserum prepared to calmodulin-(68–79) crossreacted with calmodulin in the presence of EGTA. When $CaCl_2$ was included in all the solutions, the proportion of antibody bound to calmodulin for this peptide antiserum was reduced relative to its reactivity with calmodulin in the presence of EGTA (Fig. 2*B*). Similarly, antiserum elicited by calmodulin-(80–92) bound calmodulin when EGTA was present, but bound calmodulin less efficiently in the presence of $CaCl_2$ (Fig. 2*C*). Antiserum to calmodulin-(141–148) recognized calmodulin only when EGTA was included in the reaction buffer (Fig. 2*D*). The inability of antibody to react with regions of the calmodulin molecule in the presence of $CaCl_2$ could reflect a conformationally directed binding of $Ca^{2+}$-saturated or apocalmodulin to the polyvinyl chloride surface of the microtiter well. This possibility was tested by coating the wells with calmodulin in the presence of $Ca^{2+}$ and continuing the experiment in the presence of EGTA. The converse experiment was also performed. The results (not shown) were indicative of a random binding process of calmodulin to the plastic and yielded results identical to those observed as if the entire experiment had been performed solely in EGTA or $Ca^{2+}$.

**Fluid-Phase Assays with the Peptide Antisera Detect Four Cognate Sequences That Undergo Calcium-Dependent Changes in Antigenicity.** The reactivities of peptide antisera were also analyzed by a solution-phase immunoprecipitation assay. This assay uses Bolton–Hunter-labeled calmodulin and is similar to the one described by Chafouleas *et al.* (20). The iodinated calmodulin is as efficient as the native protein in stimulating phosphodiesterase activity (20) and thus closely approximates the structure of the unlabeled molecule.

The crossreactivities of the four peptide antisera to calmodulin are shown in Fig. 3. Protein A-Sepharose plus preimmune sera or buffer without sera did not precipitate labeled calmodulin in the presence of $Ca^{2+}$ or EGTA (data not shown), indicating that nonspecific precipitation of the calmodulin did not occur. All four peptides elicited antisera that precipitated iodinated calmodulin in a concentration-dependent fashion when EGTA was included in the buffer (Fig. 3). In contrast, none of the antisera significantly precipitated $Ca^{2+}$-saturated calmodulin. The $Ca^{2+}$-dependent changes in the antigenicity of calmodulin (depicted in Fig. 3) for the serum of individual rabbits were confirmed with antisera to calmodulin-(9–19), -(68–79), and -(80–92) from one to two additional rabbits (data not shown).

## DISCUSSION

Many proteins exist in more than one conformational state depending on the presence of ions, cofactors, or post-translational modifications. The regions of a protein that undergo conformational transitions can readily be assessed only if the crystal structure has been determined for each conformational state. This study examined another strategy for identifying regions that undergo changes in conformation that is rapid, sequence-specific, and applicable to proteins for which the crystal structure has not been solved. Peptide antibodies were used as reporter molecules to localize conformational changes to the cognate sequence in the folded protein. This approach is useful providing two conditions are satisfied. First, the peptide antibody should bind the protein in only one of two or more conformational states. Second, the cognate sequence should be solvent-exposed and, therefore, sterically accessible to specific antibody in each conformational state. The latter requirement seeks to exclude loss of antigenic reactivity as a result of changes in tertiary or

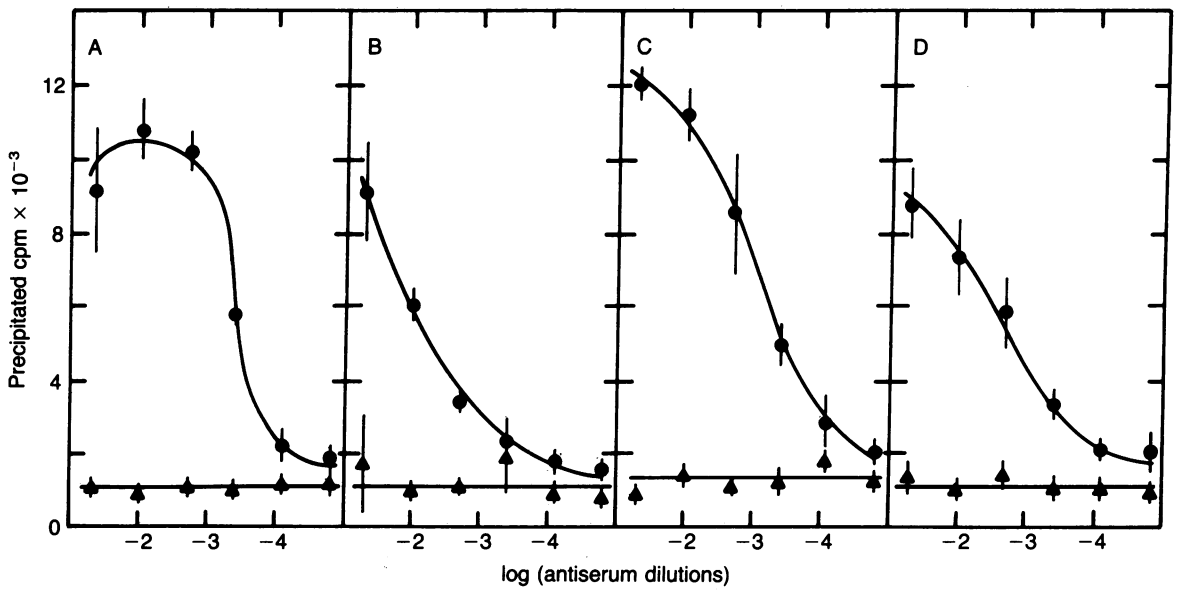


FIG. 3. Effect of EGTA and calcium on the ability of peptide antisera to immunoprecipitate calmodulin in fluid phase. (*A*) Crossreactivity with calmodulin-(9–19) antiserum. (*B*) Calmodulin-(68–79) antiserum. (*C*) Calmodulin-(80–92) antiserum. (*D*) Calmodulin-(141–148) antiserum. Presence of EGTA (●); presence of calcium (▲). Each point represents the average ± SD of assays performed in triplicate. The results depict data obtained with the antiserum from a single immunized rabbit and are representative of other animals immunized with the same peptide-carrier conjugate.

quaternary structure that bury the cognate sequence in the interior of the protein.

Calmodulin was chosen for this study because (*i*) it undergoes a well-defined calcium-induced conformational transition (7, 8, 19); (*ii*) the change in conformation is biologically important since only the $Ca^{2+}$-saturated form is functionally active (9, 10); and (*iii*) the x-ray crystal structure of $Ca^{2+}$-saturated calmodulin has been determined (11). Consequently, the secondary structures and surface accessibilities of the cognate sequences can be correlated with their antigenic reactivity in the presence of $Ca^{2+}$.

Antisera were prepared to four peptides corresponding to α-helical regions in $Ca^{2+}$-saturated calmodulin. The crossreactivities of each peptide antiserum with calmodulin were analyzed using solid- and solution-phase assays. Both assays were used to evaluate the potential artifacts inherent to each monitoring method. On the one hand, the antigenicity of specific regions of calmodulin bound to a solid support might be reduced due to the inaccessibility of these regions to the corresponding antibody. Conversely, the antigenicity of other regions of solid-phase calmodulin might be increased by the protein's interaction with the plastic surface, owing to partial denaturation and unfolding of the molecule and the exposure of normally hidden epitopes. On the other hand, the iodination of calmodulin for solution phase assays necessarily introduces nonnatural groups, and these modifications could alter an epitope directly or indirectly by disrupting the secondary structure of a cognate sequence. Accordingly, the results of both assays were considered in the interpretations of the experiments.

Three of the four synthetic calmodulin peptides elicited antisera that bound the apoprotein more strongly than the $Ca^{2+}$-saturated conformer in the solid-phase immunoassay. All four peptide antisera reacted exclusively with apocalmodulin in the solution-phase assay. The structural basis for the $Ca^{2+}$-induced loss of antigenic crossreactivity was sought by reference to the x-ray crystal structure of calmodulin. In the presence of calcium, the regions that correspond to calmodulin-(9–19), -(68–79), -(80–92), and -(141–148) are solvent exposed and α-helical (11). The susceptibility of lysine-77 of calmodulin to trypsin cleavage in the presence of $Ca^{2+}$ (21) provides additional evidence that residues 68–79 are accessible to macromolecules such as proteases. By contrast, the secondary structures and surface accessibilities of these regions in the apoprotein have not been determined. Based on the relative resistance of the central helix to tryptic digestion in the absence of calcium, Babu *et al.* (11) proposed that this region may be buried in the apoprotein. However, its crossreactivity with antisera to calmodulin-(68–79) and -(80–92) (Figs. 2 and 3) indicates that the crossreactive epitopes in this region are also surface exposed in the absence of calcium. Similarly, the cognate sequence corresponding to residues 141–148 is antigenic in the absence of calcium and, therefore, surface exposed in the apoprotein. Antibody elicited to calmodulin-(9–19) showed no crossreactivity to calmodulin when analyzed by the solid-phase assay (Fig. 2*A*). However, in solution a $Ca^{2+}$-dependent reactivity was observed (Fig. 3*A*). This discrepancy probably reflects an artifact of the solid-phase assay system (discussed above), as a result of the inaccessibility of antibody to the crossreactive epitope. It is unlikely that this region of calmodulin suffers from a local alteration in its structure due to the addition of the iodinated Bolton–Hunter reagent to free amino groups because (*i*) the N-terminal alanine is acetylated (18) and would not react with this reagent and (*ii*) the results from the trypic digestion of calmodulin (22) as well as the chemical modification of its lysine side chains in the presence of calcium (18) suggest that at most two lysines in calmodulin (particularly lysine-77) are derivatized by this reagent during the procedure used in these experiments. Therefore, it is likely that the solution-phase assay result is correct and that the crossreactive epitope recognized by antiserum to calmodulin-(9–19) is also surface exposed in apocalmodulin. Similarly, if a significant population of calmodulin molecules are denatured upon binding the plastic matrix, then a partial recognition of randomly exposed regions of calmodulin by sequence specific antibodies might occur, even in the presence of calcium. This denaturation may explain the residual antigenicity of calmodulin-(68–79), and to a smaller extent, calmodulin-(80–92) of $Ca^{2+}$-saturated calmodulin in the solid-phase assay but not in the solution-phase assay. Accordingly,

we conclude that each of the four monitored regions exhibits $Ca^{2+}$-dependent changes in antigenicity.

From the crystal structure of calcium-saturated calmodulin and the crossreactivity with peptide antisera in the absence of calcium, it is probable that all four regions are surface exposed in both conformational states. It follows that the loss of crossreactivity is most likely due to $Ca^{2+}$-induced changes in the local structure of the monitored regions. The structural details of these conformational changes were not addressed in this study. However, the increase in the $\alpha$-helical content of calmodulin that is associated with calcium binding and the apparent preference of peptide antibodies for highly mobile regions of proteins (2) could indicate that $Ca^{2+}$-induced, local transitions occur in the cognate sequences from antigenic, flexible structures to the less antigenic relatively ordered helices that are evident from the x-ray crystal structure of $Ca^{2+}$-saturated calmodulin.

It has long been observed that antibodies to polypeptide fragments can be used to distinguish native from random conformations of the same protein (23–26). As a result, these reagents have been employed in protein-folding studies (23–26). The experiments reported here describe how antisera to small synthetic peptides may be used to monitor local conformational changes in proteins that undergo ligand-induced transitions between two native but structurally and functionally distinct states. Providing the cognate sequence is sterically accessible to the binding sites of antibody molecules in both states but antigenic in only one, peptide antibodies can serve as sequence-specific reporter groups that monitor changes in local structure. Insofar as peptide antibodies preferentially bind flexible segments of proteins (2), the use of these reagents as structural probes may be restricted to regions of proteins that undergo transitions from mobile to constrained conformations.

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