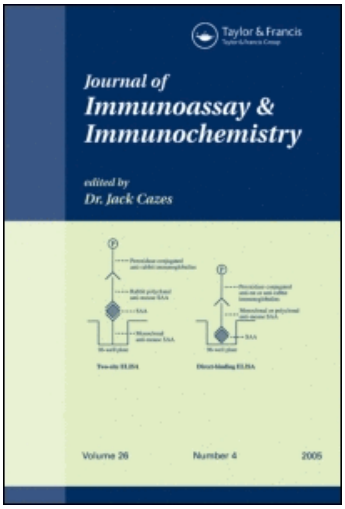

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CHARACTERIZATION OF A CALMODULIN ANTISERUM BY ITS REACTIONS WITH FRAGMENTS OF THE CALMODULIN MOLECULE

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ABSTRACT

A high affinity antibody, specific to the calcium-free form af calmodulin, which had previously been developed using N-acetyl-muramyl-L-alanyl-D-isoglutamine-calmodulin conjugate as an immunogen, was tested for cross-reactivity with tryptic fragments of calmodulin (CaM_{1-77}, CaM_{1-90}, CaM_{78-149}, and $CaM_{106-149}$) as well as with synthetic peptides corresponding to the lst, 2nd, and 3rd calcium binding loop of calmodulin. The results showed that the antigenic determinant involves a special conformation of amino acid residues 90-106 in the 3rd calcium-binding domain.

(KEY WORDS: calmodulin, antibody specificity)

INTRODUCTION

In ref. 1 we have described preparation of a high affinity antibody that was specific to the calcium-free form of calmodulin. In other words, the antigenic determinant reacting with the antibody changes its conformation when binding calcium to such an extent that it is not recognized by the binding sites of the antibody. The specificity

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of the antiserum is therefore different from that described by van Eldik et al. (2) and by Harper (3). In this paper we studied the antigenic determinant of calmodulin reacting with the antibody using 4 proteolytic fragments of calmodulin and 4 synthetic peptides corresponding to different parts of the calmodulin molecule.

MATERIALS AND METHODS

Calmodulin was prepared by the method of Gopalakrishna and Anderson (4) with modifications described in ref. (5). Tryptic calmodulin fragments were isolated as described in ref. (5) and the digestion was performed according to Drabikowski et al. (6) and Walsh et al. (7). The purity of fragments was checked by overloaded urea polyacrylamide gel electrophoresis (not shown), amino acid analysis and additionally by cAMP phosphodiesterase (PDE) assay. Newton et al. (8) have demonstrated that none of the calmodulin fragments could activate PDE. Consequently, if there was any activation of PDE by a fragment preparation, it should be due to calmodulin contamination. We have observed only slight activation of PDE by CaM_{1-77} and CaM_{78-149} fragments at concentrations 400 times higher than the concentrations of calmodulin causing half maximal activation. Fragment CaM_{1-90} did not cause any PDE activation when used at the same concentration. However, according to urea gel electrophoresis and amino acid analysis the preparation of CaM_{1-77} was slightly contaminated with fragment CaM_{78-149} ($\lesssim 2\%$).

Synthetic peptide corresponding to the 1st binding loop (CaM_{20-31}) was kindly supplied by Dr. Boris and peptides corresponding to the 2nd (CaM_{54-69}) and 3rd (CaM_{91-106} linear and CaM_{91-106} cyclic) binding loops by Drs. Buchta, Bondi and Friedkin. Their qualities were the same as de-

scribed in (9) and (10) respectively. ^{125}I-Calmodulin (128 μCi/μg was purchased from New Engl. Nuclear. Anti-calmodulin serum was that described in (1).

Peptides were tested in the concentration range 10^{-9} - 10^{0} mg/ml. The radioimmunoassay system was the same as described in (1). In brief: 0.1 ml of the relevant peptide or calmodulin solution was added to 0.25 ml of the antiserum solution (1:10 000). Finally, 0.1 ml of ^{125}I-Calmodulin was added (appr. 5000 cpm). 0.1 M Tris buffer, pH 7.8 containing 0.1 mM EGTA and 0.2% BSA were used for making solutions of all substances. However, the extremely hydrophobic peptide CaM_{91-106} was first dissolved in dimethylsolfoxide and then further in the above mentioned buffer. The 48 hour incubation was followed by a 16 h incubation with the second antibody. After recalculation of the concentrations in mol per litre, the relative displacement factor (RDF) was enumerated for all the substances as 100 x $c^{CaM}_{50\%}/c^{x}_{50\%}$.

RESULTS AND DISCUSSION

The results are summarized in Table I and illustrated in Fig.1. Fig. 2 shows the regions of the calmodulin molecule corresponding to the different fragments and synthetic peptides tested. As can be seen from Table I, only two fragments tested display some degree of cross-reactivity, i.e. the tryptic fragments obtained by limited digestion in the presence of calcium. The C-terminal fragment CaM_{78-149} displays full cross-reactivity and the N-terminal fragment CaM_{1-77} displays 0.86% of the immunoreactivity of calmodulin. The two fragments obtained by tryptic digestion in the absence of calcium (CaM_{1-90} and $CaM_{107-149}$) showed no cross-reactivity. These facts would point to the amino acid sequence 90-106 in the 3rd calcium binding domain being an

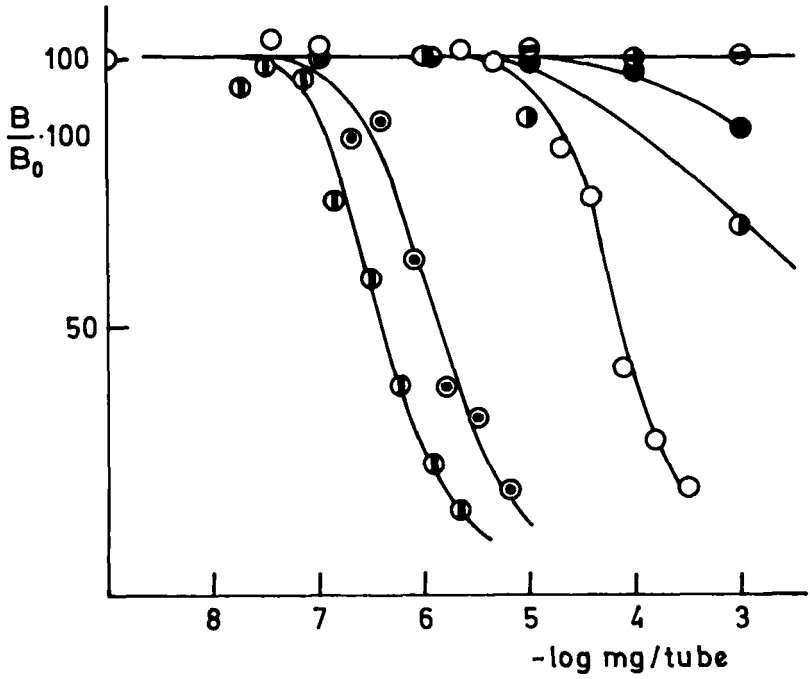

FIGURE 1.
Curves showing inhibition of iodinated calmodulin binding to calmodulin-antibody by the following peptides: ⊙ calmodulin, ○ CaM_{1-77}, ◐ CaM_{78-149}, ◑ $CaM_{107-149}$, ⊖ CaM_{20-31}, and ● CaM_{1-90}.

antigenic determinant in our case. It also points to the importance of the conformational characteristics of the antigenic determinant. To support this finding, knowledge of the cross-reactivity of other fragments (e.g. CaM_{78-125}) might contribute. However, such fragments cannot be obtained by tryptic digestion and synthesis of such long peptides is not an easy matter.

The experiments concerning the tryptic digestion of calmodulin (7) in the presence or absence of calcium ions suggest that the third calcium binding domain is exposed to the environment in the absence of calcium ions and hidden in their presence.

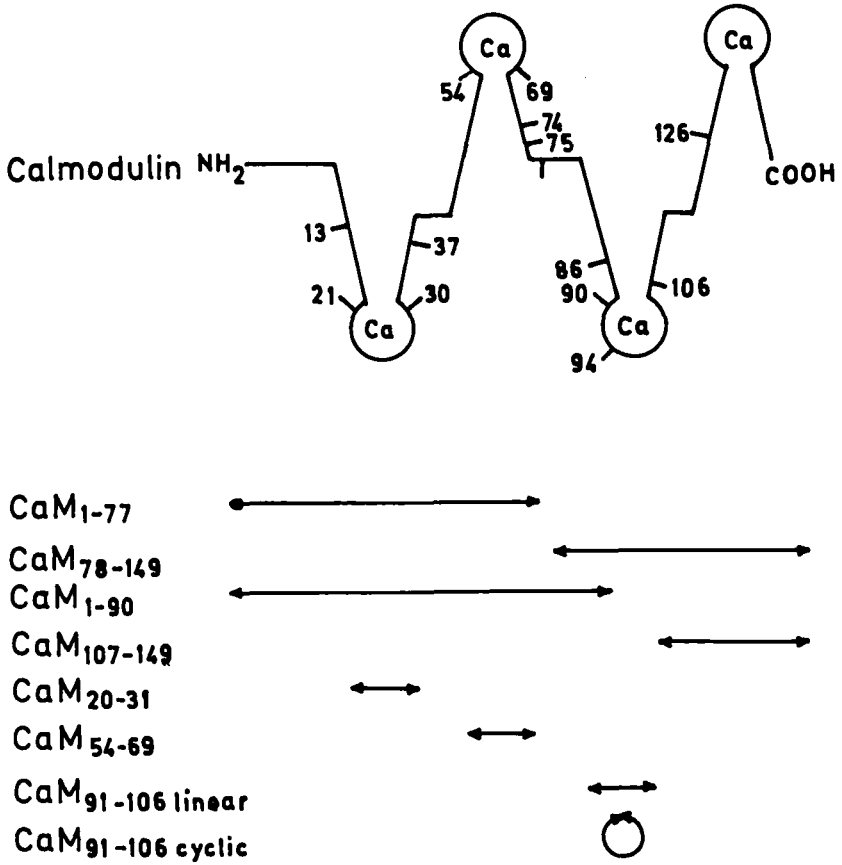

FIGURE 2.
Survey of the parts of calmodulin corresponding to the relevant peptides studied.

This fits with our finding that the antibody reacts only with the calcium-free form of calmodulin (1). The results using the synthetic peptides (none of them, even the CaM_{91-106} showing any degree of cross-reactivity) further stress the significance of the conformation. Peptide fragments of synthetic peptides are invariably largely unfolded. The "correctly"folded form with which the unfolded form

TABLE I

Cross-reactivity of tryptic (t) and synthetic (s) peptides with a calmodulin-antiserum. Peptides in the (t) group were prepared by limited proteolysis in the presence of Ca^{2+} (a) and EDTA (b), respectively.

	Substance	Relative displacement factor (RDF)
	Calmodulin	100
(t)	CaM_{1-77}[a]	0.86
	CaM_{78-149}[a]	112.8
	CaM_{1-90}[b]	< 0.04
	$CaM_{107-149}$[b]	< 0.04
(s)	CaM_{20-31}	< 0.002
	CaM_{54-69}	< 0.002
	CaM_{91-106}linear	< 0.002
	CaM_{91-106}cyclic	< 0.002

is in equilibrium may represent as little as 1 part in 10^4 or 10^5 of the peptide. The association constant for antibody binding to the fragment is therefore attenuated by a certain factor (11). The cross-reactivity of the cyclic peptide CaM_{91-106} cyclic is, however, only a rough approximation because of the extreme hydrophobicity of this peptide. No check was made of the real concentration of the compound in the tested solutions. The 0.86% immunoreactivity of the N-fragment CaM_{1-77} prepared in the presence of calcium may be explained by traces of CaM_{78-149} fragment contaminating the preparation (see Materials and Methods).

Van Eldik et al. (2) reported on an antibody specific to the 137-143 amino acid sequence obtained using performic acid oxidized calmo-

dulin. The conformation of the antigenic determinant was not important in their case as the antibody reacted with the calcium-bound and calcium-free forms as well as with the synthetic peptides corresponding to the relevant amino acid sequence. Katajima et al. (12) showed that antibodies produced using performic acid-oxidized calmodulin or dinitrophenylated calmodulin have less affinity to native calmodulin than to modified calmodulin. They have obtained antibodies of better affinity using native calmodulin together with methylated albumin as immunogen. Biber et al. (13) recently found that antisera elicited in rabbits by performic acid-oxidized calmodulin only bind to the chloramin-treated calmodulin or performic acid-oxidized calmodulin but not to untreated calmodulin. The fact that one can obtain antisera which recognize only the calcium-bound form of calmodulin has been shown by Harper (3). Two classes of such antisera were found, distinguishable by the concentration of calcium needed for the half -maximal binding. The development of antibodies against different regions of the calmodulin molecule and with other differences in their properties may be of value in studies of relations between the structure of calmodulin and its functions (14).

We conclude from our studies that using CaM-MDP conjugate a very specific and high affinity antibody was obtained demanding the undisturbed calmodulin molecule, with no calcium bound, for its binding and seeing the region involving amino acid residues 90-106 in the calmodulin molecule.

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