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Mapping of Calmodulin-binding Domain of Ca^{2+}/calmodulin-dependent Protein Kinase II from Rat Brain

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Recent molecular cloning experiments have identified a 25 amino-acid region as the calmodulin-binding domain of the α-subunit of rat brain Ca^{2+}/calmodulin-dependent multifunctional protein kinase II (CaM-K II). Synthetic peptides, derived from the deduced amino-acid sequence encompassing this region, were examined for their ability to bind calmodulin in a calcium dependent manner and to inhibit the Ca^{2+}/calmodulin-dependent autophosphorylation of CaM-K II. Comparison of these structure-function relationships highlighted a region of 5 amino-acids, which was essential for calmodulin interaction and inhibition of kinase activity. This region demonstrated some homology with other calmodulin-binding peptides, and may represent a key site of interaction of the kinase with calmodulin. These analyses provide additional insight into the molecular mechanism underlying the Ca^{2+} regulation of CaM-K II.

Many of the intracellular actions of calcium are mediated via the ubiquitous calcium-binding protein, calmodulin (CaM). A number of CaM-regulated enzymes, with distinct structure and function, have been identified. The molecular mechanism by which CaM regulates its target proteins has been examined in a variety of laboratories (1-5). The potential to form amphiphilic α-helices has been suggested as the common property of CaM-binding peptides, which show only limited homology in primary sequences. Peptides modeled on the CaM-binding domains of several enzymes have demonstrated both Ca^{2+}-dependent CaM-binding and the potential to form amphiphilic helices. Whether this structural feature will be present in all CaM-regulated enzymes can only be speculated.

Recent work characterizing the protein-chemical and immunological properties of Ca^{2+}/CaM-dependent multifunctional protein kinases has indicated the existence of a family of these enzymes in mammalian tissues (6). Molecular cloning of the α subunit of CaM-K II from rat brain has defined a region of the protein containing the CaM-binding domain (7). A synthetic peptide corresponding to a 20 amino-acid sequence within this domain has also been shown to bind CaM in a Ca^{2+}-dependent manner. The current study, using a variety of synthetic peptides, further defines this CaM-binding domain in CaM-K II from mammalian brain and contrasts the

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structural features in other CaM-binding peptides to gain further insight into the molecular basis of Ca^{2+}/CaM-regulation of CaM-K II.

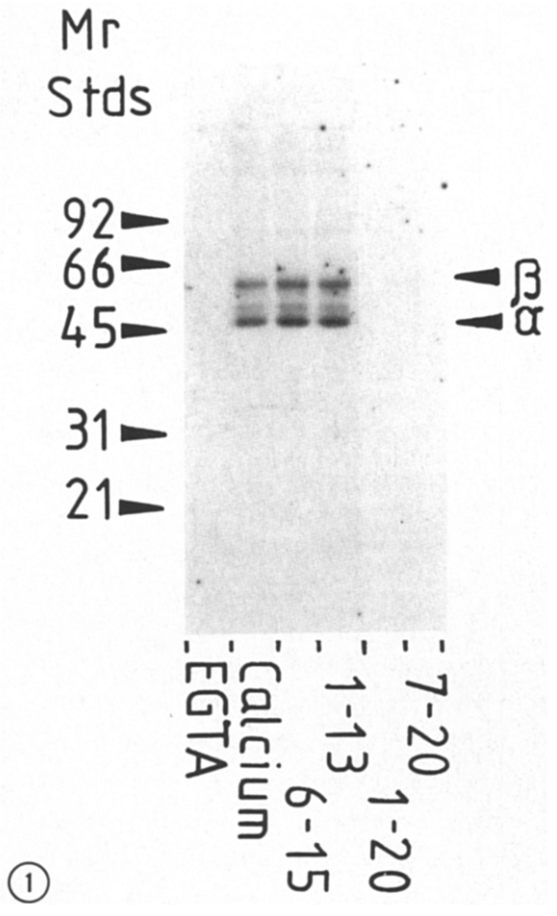
METHODS

CaM-K II was purified to near homogeneity from rat brain as previously described (8). CaM was purified from rabbit skeletal muscle (12). The autophosphorylation of CaM-K II (5.4 nM) was carried out in the presence of 100 nM CaM with 0.1 mM $CaCl_2$ (in the presence or absence of the synthetic peptides) or with 10 mM EGTA at 30°C for 1 min (6). The assays were terminated by incubation with 1% SDS at 90°C for 2 min. The phosphorylated proteins were subjected to electrophoresis on 7-16% gradient polyacrylamide gels according to Laemmli (9). The phosphate incorporated into the α (50 kDa) and β (60 kDa) subunits was determined by counting the gel slices containing these proteins, visualized by staining with Coomassie blue and by autoradiography. Synthetic peptides modeled after the CaM-binding domain of CaM-K II (7) were prepared by the Merrifield solid-phase procedure (10). Binding of peptides to CaM in the presence of 1mM $CaCl_2$ or 10 mM EGTA was monitored as mobility shifts on electrophoresis in 7.5 % slab gels according to (9), omitting SDS or in 10% gels in the presence of 8M urea (11).

RESULTS

Purified CaM-K II demonstrated rapid autophosphorylation of both α and β subunits, in the presence of Ca^{2+} and CaM (figure 1). No phosphate was incorporated in the presence of EGTA. Ka for CaM for the autophosphorylation of either α or β-subunits was determined to be indistinguishable at approximately 100 nM under our assay conditions. Thus at limiting CaM concentrations (100 nM) in all assays, effects of varying concentrations of synthetic peptides were examined. The peptide, designated 1-20, represents the region from residues 290 to 309 in the α-subunit (from the deduced amino-acid sequence obtained from the full-length cDNA for the 50 kDa subunit - Ref.13), previously shown to contain the CaM-binding domain. The other peptides, modeled around this region, were termed 1-13, 6-15 and 7-20. At 1 μM concentration 1-20 and 7-20 completely inhibited autophosphorylation of both the 50 and 60 kDa polypeptides (figure 1), to the same extent seen with 10 mM EGTA. In contrast, peptides 1-13 and 6-15 (at 1 μM) were without effect. Dose-response curves for the inhibition of Ca^{2+}/CaM-dependent autophosphorylation of CaM-K II indicated K_i values for peptides 1-20 and 7-20 between 50 and 70 nM (figure 2). Peptides 1-13 and 6-15 had no effect even at concentrations up to 10 μM. Although only the inhibition curves for phosphorylation of the 50 kDa subunit are shown, inhibition of the β-subunit (60 kDa) phosphorylation by these peptides demonstrated identical dose-response curves to those seen for the α-subunit (50 kDa). Inhibition of autophosphorylation by the peptides (at 1μM) could be overcome by addition of 5μM CaM to all assays. In addition, none of the peptides (at 10μM) were significantly phosphorylated in assays, carried out according to Kemp et al (1). This suggests that the inhibition was competitive with respect to the activator, CaM, and the peptides acted primarily as CaM-antagonists in these assays.

To examine the direct interaction of the peptides with CaM, CaM (4 μg) was subjected to electrophoresis in non-denaturing 7.5% gels in the presence of 1 mM $CaCl_2$ or with 10 mM EGTA (figure 3A). In the presence of calcium, peptides 1-20 and 7-20, at a molar ratio of 2:1 with CaM, retarded the mobility of CaM, indicating an association with the peptides. No change in CaM mobility was observed in the presence of EGTA. In comparison, 1-13 and 6-15 failed to bind CaM



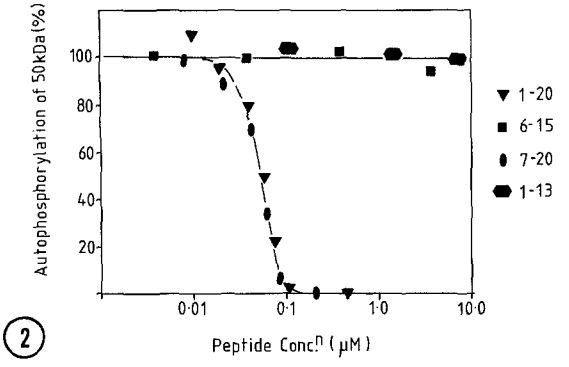


Figure 1. Inhibition of Ca^{2+}/CaM-dependent Autophosphorylation of CaM-kinase II by Synthetic Peptides.

CaM-kinase II (5.4 nM) was autophosphorylated in the presence of EGTA and calcium as described in the methods section. Inhibition by synthetic peptides (1 μM) was carried out in the presence of calcium. All reactions contained calmodulin (100 nM).

Figure 2. Inhibition of Ca^{2+}/CaM-dependent Alpha-subunit Autophosphorylation by Peptides.

Autophosphorylation was carried out as described for figure 1. The Ca^{2+}/CaM-stimulated autophosphorylation of the α-subunit (50 kDa) in the absence of added peptide was set as 100%.

in the presence or absence of calcium. We have previously shown that the Ca^{2+}-dependent association of CaM with its binding proteins was stable even in the presence of 8M urea (11). In an analogous manner, peptides 1-20 and 7-20 (at a molar ratio of 1:1 with CaM) all showed Ca^{2+}-dependent association with CaM in the presence of 8M urea (figure 3B). The binding of peptides resulted in the increased mobility of CaM in 10% urea-gels. Approximately 50% of the CaM showed the mobility shift with the remainder migrating with free CaM (control lane - C). Increasing peptide concentrations (to molar ratios of 2:1, 5:1 or 10:1 with CaM) led to increased mobility of all the CaM. The protein staining bands on the urea-gels were much sharper than those seen on the native gels, permitting better quantitative analysis of peptide-CaM interactions by soft-laser densitometry. The data suggested a Ca^{2+}-dependent interaction of the peptides with CaM

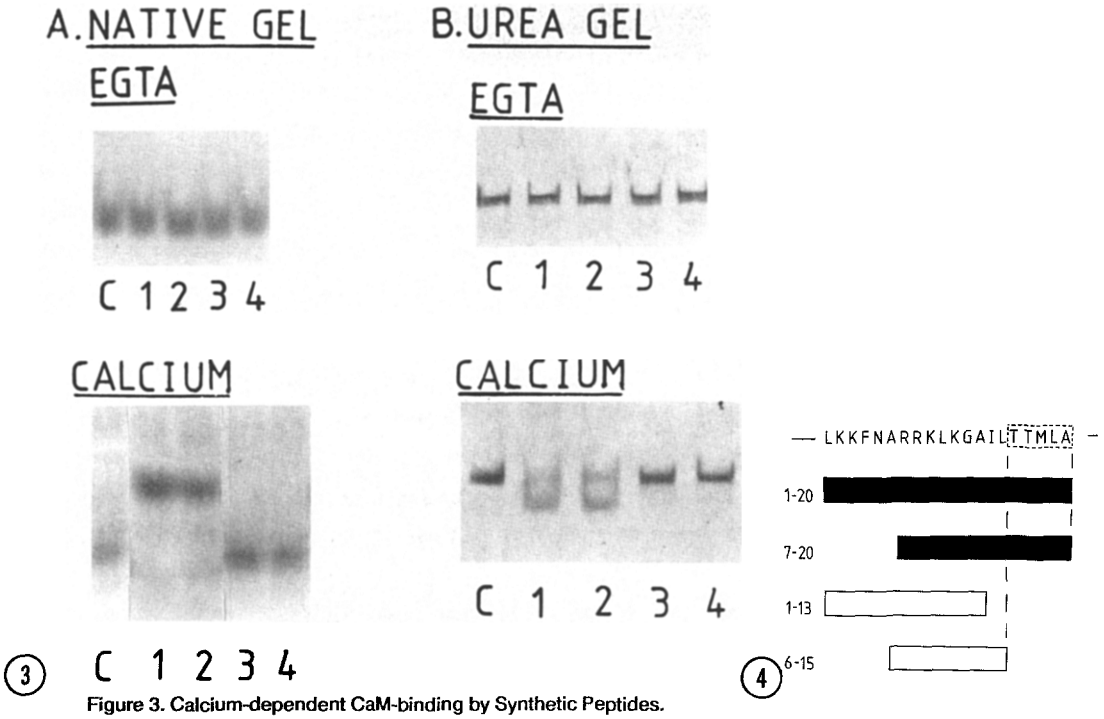


Figure 3. Calcium-dependent CaM-binding by Synthetic Peptides.

Binding of CaM (4 μg) to synthetic peptides was examined in the presence of EGTA and calcium using electrophoresis in 7.5 % non-denaturing ("native") gels (panel A) at a peptide to CaM molar ratio of 2:1 and in 10 % polyacrylamide gels in the presence of 8M urea (panel B) at a molar ratio of 1:1. The lanes are marked as follows: CaM alone - C; lane containing CaM with peptide 1-20 - 1; peptide 7-20 - 2; peptide 1-13 - 3; and peptide 6-15 - 4.

Figure 4. Amino-acid Sequences of Active and Inactive Peptides Corresponding to the CaM-binding Domain of CaM-kinase II.

Region of CaM-binding domain (designated by amino-acid sequence, "1-20") chosen for the synthesis of peptides is shown. The "active" peptides which inhibited Ca^{2+}-stimulated autophosphorylation of CaM-kinase II and demonstrated Ca^{2+}-dependent binding to CaM are shown as solid bars. The "inactive" peptides are represented by the open bars.

with an approximate molar stoichiometry of 1:1. In comparison, peptides, 1-13 and 6-15, failed to demonstrate any CaM-binding.

Comparison of the amino-acid sequences of the CaM-binding peptides (figure 4) indicated that the minimal peptide required for CaM-binding, as determined by either the inhibition of autophosphorylation of CaM-K II or by mobility shifts of CaM on electrophoresis, was represented by the 13 residue peptide, 7-20. This region was included in both "active" peptides. In contrast, the peptide 1-13, which differed from 1-20 only in the absence of COOH-terminal 7 residues was inactive, suggesting an important functional role for residues 14-20 in CaM-binding. Comparison of peptides 7-20 (active) and 6-15 (inactive) provide further argument for a restricted region, namely 16-20, for the Ca^{2+}-dependent association with CaM. In preliminary studies, K_d for association of peptide 7-20 with CaM was determined by the method of Cox et al (2) to be between 0.1 and 0.3 nM. In comparison, Kd for 6-15 could not be accurately estimated being greater than 10 μM. Thus, the sequence represented by residues 16-20 appears to be critical for the high affinity binding to CaM.

DISCUSSION

Many of the intracellular actions of calcium are mediated by the calcium-binding protein, calmodulin (CaM). In response to elevated cellular calcium, CaM activates a variety of target proteins (14). The manner in which CaM regulates the many "acceptor proteins" with differing structure and function has been the focus of research in many laboratories. Interaction of CaM with a number of amphiphilic peptides, such as the 26 amino-acid cytotoxic peptide from bee venom, mellitin, has provided useful information about the structural features for a "CaM-binding domain" (2). Such studies suggest a role for the potential amphiphilic α-helix structures encompassed by all CaM-binding peptides. A CaM-binding fragment of skeletal muscle myosin light chain kinase showed considerable homology with CaM-binding peptides, such as mellitin (15). Use of synthetic peptides corresponding to the proposed CaM-binding domains for smooth muscle (3) and skeletal muscle (4) myosin light chain kinases provided strong evidence for limited sequences, which could form amphiphilic α-helices, as a common determinant in the binding of CaM.

Current report on mapping of the CaM-binding domain of Ca^{2+}/CaM-dependent multifunctional protein kinase II (CaM-K II) extends these studies to an oligomeric enzyme showing very different structure and substrate specificity to the myosin light chain kinases. Four new synthetic peptides, highlighting the CaM-binding domain of CaM-K II (7), were generated to examine the structural requirements for Ca^{2+}-dependent CaM-binding. Peptides were analyzed for their potency as CaM-antagonists by the Ca^{2+}/CaM-dependent autophosphorylation of CaM-K II from rat brain and for direct association with CaM in mobility shift analyses in native and urea gels. The data indicated that the peptide termed 7-20, representing residues 297-309 in the α-subunit of CaM-kinase II, as the smallest CaM-binding domain. Elimination of residues 16 to 20 in peptide 6-15 abolished CaM-antagonist activity and binding, suggesting that these five residues played an important role in the Ca^{2+}-dependent high affinity binding to CaM. This sequence, also found in the β-subunit of CaM-K II, inhibited the CaM-stimulated autophosphorylation of both α and β-subunits in identical manner.

Extensive autophosphorylation of CaM-K II will result in an enzyme, which is essentially Ca^{2+}/CaM-independent (17). This form of the enzyme is particularly useful in the investigation of potential inhibitory or "pseudosubstrate" domain (1, 4) in this protein kinase. Experiments by Payne et al (16) have used the Ca^{2+}/CaM-independent form of CaM-K II to examine the ability of synthetic peptides to inhibit exogenous substrate phosphorylation. These studies indicate that peptides, 1-20, 1-13, 7-20 and 6-15, all contain the structural features necessary for inhibition of the substrate phosphorylation of the synthetic peptide, syntide-2 (modeled on site-2 of glycogen synthase). These studies also observed that the synthetic peptides did not inhibit the phosphorylation of protein substrates, such as glycogen synthase or casein. Hence, the ability of the peptides to compete with substrates appears to complex and may vary with the substrate. Using Ca^{2+}/CaM-stimulated cyclic nucleotide phosphodiesterase, the CaM-antagonism was seen with peptides 1-20 and 7-20. Peptides 1-13 and 6-15 were not inhibitory in this assay. They interpreted the data to indicate that the substrate-directed inhibitory domain and CaM-binding domain showed probable overlap.

In contrast, the current studies have focused specifically on the ability of synthetic peptides to compete with CaM and block the Ca^{2+}/CaM-dependent autophosphorylation of CaM-K II, a process implicated in the physiological auto-regulation of the enzyme activity in intact tissue (17). The simple expediency of using the autophosphorylation assay, containing only CaM and CaM-K II, enabled us to identify the CaM-binding peptides. Autophosphorylation was limited to low stoichiometry - less than 1 mol per mol of holoenzyme (maximal phosphorylation can introduce up to 25 moles of phosphate into the holoenzyme), to focus only on the Ca^{2+}/CaM-dependent process (17). In such analyses, only peptides 1-20 and 7-20 were potent CaM-antagonists (K_i 50 to 70 nM). Peptides 1-13 and 6-15 were ineffective. The present study has also established that 1-20 and 7-20 demonstrate direct Ca^{2+}-dependent CaM-binding. Hence, the specificity associated with peptides 1-20 and 7-20 reflects their interaction with CaM and not the target enzymes. In summary, the substrate antagonism demonstrated a distinct peptide specificity and much lower potency (i.e. 25 to 200 μM) than that seen in our studies of CaM-antagonism. Hence, distinct structural determinants are involved in the association with CaM, compared with those involved in inhibition of substrate phosphorylation.

Comparison of the primary sequence of peptide, 7-20, with other CaM-binding peptides, such as mellitin, M-13 (15), or RS-20 (3) indicates some homology specifically in the region of the five amino-acid residues, which enclose the most hydrophobic region of the peptide (figure 5). This "hydrophobic" sequence also shows homology with other CaM-binding peptides investigated by Cox et al (2). The peptide representing the NH_2-terminal 11-residues of M-13 retaining four of the five amino-acids in the designated "hydrophobic" box (figure 5) has been shown to act as a CaM-antagonist (1). This data does not discount the importance of the amphiphilic domain, as elimination of 2 of the four arginines in RS-20 can reduce the K_d for CaM-binding by 8-fold (3). Peptide 7-20 showed a particularly striking homology with mellitin in the "hydrophobic" region, which would be predicted to conform to a β-sheet structure. Indeed, both 7-20 and mellitin conform poorly to the amphipathic helix, considered to be essential for CaM-binding by others, using the helical wheel patterns (2,3). This could suggest that the interaction of these peptides with CaM might be different from those seen with M-13 and RS-20, which better fulfill the requirements for a amphipathic helix, implicated in the putative helix-helix association involving the interdomain helix in CaM.

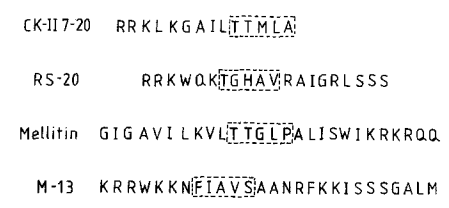


Figure 5. Comparison of Calmodulin-binding Peptides.

Primary sequences of other CaM-binding peptides, e.g. Mellitin (2), RS-20 (representing CaM-binding domain of smooth muscle myosin light chain kinase - 3), M-13 (from skeletal muscle myosin light chain kinase) are compared with that of the minimal active peptide 7-20 from CaM-kinase II (CK-II). The blocks of five amino-acids representing the most hydrophobic region in each peptides are outlined.

NMR analysis of the interaction of mellitin with deuterated-CaM (18) has indicated that while the major binding site for mellitin was predicted to lie in the COOH-terminal half of CaM, binding of mellitin induced perturbations in both NH_2- and COOH-halves of CaM. This suggests that binding of the peptide induces extensive changes in CaM structure. Extension of these studies with two equal fragments of mellitin established that the NH_2-terminal 13 residues of mellitin accounted for the spectral changes seen in the NH_2-terminal half of CaM, while the fragment 14-26 of mellitin, containing some of the "hydrophobic block" and majority of the basic residues, interacted with the COOH-terminal of CaM (19). Based on structural comparison with mellitin, peptide 7-20 might be expected to associate primarily with the COOH-terminal half of CaM. Furthermore, our data would suggest that while the α-helix present in 7-20 may be important in the CaM-antagonism, it is the "hydrophobic" region of five amino-acids which represents the key element in generating high affinity binding for CaM.

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REFERENCES

1. Kemp,B.E., Pearson,R.B., Guerriero,V.C., Bagchi,I.C., and Means,A.R. (1987) J. Biol. Chem. 262, 2542-2548.
2. Cox,J.A., Comte,M., Fitton,J.E. and DeGrado,W.F. (1985) J. Biol. Chem. 260, 2527-2534.
3. Lucas,T.J., Burgess,W.H., Prendergast,F.G., Lau,W. and Watterson,D.M. (1986) Biochemistry 25, 1458-1464.
4. Kennelly,P.J., Edelman,A.M., Blumenthal,D.K. and Krebs,E.G. (1987) J. Biol. Chem. 262, 11958-11963.
5. O'Neil,K.T., Wolfe,H.R., Ericksen-Viitanen, DeGrado,W.F. (1987) Science 236, 1454-1456.
6. Shenolikar,S., Licktieg,R., Hardie,D.G., Soderling,T.R., Hanley,R.M. and Kelly,P.T. (1986) Eur. J. Biochem. 161, 739-747.
7. Hanley,R.M., Means,A.R., Ono,T., Kemp,B.E., Burgin,K.E., Waxham,N. and Kelly,P.T. (1987) Science 237, 293-297.
8. Kelly,P.T. and Shenolikar,S. (1986) Methods Enzymol. 139, 690-714.
9. Laemmli,U.K. (1970) Nature 227, 680-685.
10. Hodges,R.S. and Merrifield,R.B. (1975) Anal. Biochem. 65, 241-272.
11. Shenolikar,S., Cohen,P.T.W., Cohen,P., Nairn,A.C. and Perry,S.V. (1979) Eur. J. Biochem. 100, 329-337.
12. Grand,R., Shenolikar,S. and Cohen,P. (1981) Eur. J. Biochem. 113, 359-367.
13. Lin,C.R., Kapiloff,M.S., Durgian,S., Tatemoto,K., Russo,A.F., Hanson,P., Schulman,H. and Rosenfeld,M.G. (1987) Proc. Natl. Acad. Sci. USA 84, 9497-9501.
14. Hanley,R.M., Dedman,J.R. and Shenolikar,S. (1987) Am. J. Physiol. 21, C277-C285.
15. Blumenthal,D.K., Takio,K., Edelman,A.M., Charbonneau,H., Titani,K., Walsh,K.A. and Krebs,E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 3187-3191.
16. Payne,M.E., Fong,Y.L., Ono,T., Colbran,R.J., Kemp,B.E., Soderling,T.R. and Means,A.R. J. Biol. Chem. In Press.
17. Kelly,P.T., Licktieg,R. and Shenolikar,S. (1987) Calcium-binding proteins in health and disease (Eds. Norman,A.W., Vanaman,T.C. and Means,A.R. - Academic Press) pp.188-192.
18. Seeholzer,S.H., Cohn,M., Putkey,J.A., Means,A.R. and Crespi,H.L. (1986) Proc. Natl. Acad. Sci. USA 83, 3634-3638.
19. Seeholzer,S.H., Cohn,M., Wand,A.J., Crespi,H.L., Putkey,J.A. and Means,A.R. (1987) Calcium-binding proteins in health and disease (Eds. Norman,A.W., Vanaman,T.C. and Means,A.R. - Academic Press) pp. 360-371.