Vol. 86, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1979 Pages 1256-1265

CATION DEPENDENT CONFORMATIONS OF BRAIN CA^{2+}-DEPENDENT REGULATOR PROTEIN DETECTED BY NUCLEAR MAGNETIC RESONANCE

Kenneth Seamon
Department of Psychiatry
Washington University School of Medicine
Saint Louis, Missouri 63110

Received January 7,1979

SUMMARY

The 250MHz NMR spectrum of the brain Ca^{2+}-dependent regulator protein was examined in the absence of cations and in the presence of Ca^{2+} or Mg^{2+}. The Ca^{2+}-saturated regulator protein and Mg^{2+}-saturated regulator protein exhibited several spectral differences in the aromatic and aliphatic regions of their spectra. Certain spectral changes observed to occur upon addition of metal ions are qualitatively similar to those which have been observed in the spectrum of skeletal troponin-C. These results suggest that the large sequence homology between skeletal troponin-C and the regulator protein results in similar conformational changes due to the binding of Ca^{2+} or Mg^{2+}.

INTRODUCTION

Brain Ca^{2+}-dependent regulator protein (hereafter referred to as calmodulin) was first isolated from rat and shown to activate 3'-5'-cyclic nucleotide phosphodiesterase (1). Subsequently it has been shown that structurally similar activator proteins can be isolated from a wide variety of tissues and species (2-8). Furthermore, a number of enzyme systems have been characterized which can be activated in a Ca^{2+}-dependent manner by calmodulin. These include a detergent solubilized preparation of adenylate cyclase (9), smooth muscle myosin light chain kinase (10), an erythrocyte Ca^{2+}-Mg^{2+} ATPase (11), and a synaptosomal kinase capable of phosphorylating synaptosomal membrane proteins (12). The activation of these systems depends on the ability of the activator protein to bind Ca^{2+}. It has been shown that calmodulin can bind to phosphodiesterase (13) and adenylate cyclase (14) only in the presence of Ca^{2+}. Presumably, it is this Ca^{2+}-dependent interaction which is important for the activation by calmodulin.

Abbreviations: EGTA, ethylene glycol bis(βOaminoethyl ether)-N,N'-tetraacetic acid; TSP, trimethylsilylproprionic acid; tml, trimethyllysine.

0006-291X/79/041256-10$01.00/0

Calmodulin is an acidic, high affinity calcium binding protein with a molecular weight of ∿16,700 daltons. The amino acid sequence of brain calmodulin shows a large degree of homology to troponin-C, and, like troponin-C, four possible Ca^{2+} binding regions in the sequence can be identified (15). The binding of calcium by calmodulin causes changes in the protein's conformation as detected by a variety of chemical and spectroscopic techniques (16-20). Many of these Ca^{2+}-induced conformational changes are similar to those observed to occur upon Ca^{2+} binding to troponin-C (21-24). NMR has previously been used to study the effects of metal binding to troponin-C and has been quite useful in detecting changes caused by binding of metals at specific binding sites (25-27). In this communication, we report initial observations of the 250 MHz NMR spectra of metal-free calmodulin, Mg^{2+}-saturated calmodulin, and Ca^{2+}-saturated calmodulin. These results are compared to those previously reported for troponin-C.

MATERIALS & METHODS

Calmodulin was prepared essentially as described by Watterson et al (28). Purified calmodulin showed a single band when electrophoresed either on 10-25% SDS-urea acrylamide gradient slab gels or on a continuous 7.5% tris-glycine acrylamide gel.

NMR samples were prepared by dialyzing a solution containing ∿ 10 mg/ml of calmodulin for 24 hours against two changes (1 liter) of the following buffers: 1 mM EGTA, for the Ca^{2+}-Mg^{2+}-free sample; 1 mM EGTA, 5 mM $MgCl_2$, for the Ca^{2+}-free sample in the presence of excess Mg^{2+}; and 0.1 mM Ca^{2+} for the Ca^{2+}-saturated samples.[1] All solutions were at pH 7.5. The protein was then lyopholized and dissolved in 0.5 ml of D_2O containing 0.2 M KCl. The pH was adjusted to 7.5 with KOD or DCl. Total Ca^{2+} was determined in the Ca^{2+}-free samples by atomic absorption and was found to be less than 0.1 mole Ca^{2+}/mole calmodulin.

Proton spectra were run at 250 MHz in the correlation mode (29). Typical spectra were the average of 1000 scans with 1500-Hz sweep width, 1-s sweep time, with an exponential filtering of 0.5 Hz to reduce noice. Proton chemical shifts were measured from the HDO lock and adjusted to chemical shifts from internal TSP by adding 4.72 ppm. All spectra were taken at 23°C.

RESULTS

The aromatic region of the spectrum of metal-free calmodulin is shown in Figure 1A. This region of the spectrum contains resonance contributions from

[1] Published binding studies (5,18) indicate that in the presence of 0.1 mM-free Ca^{2+} calmodulin will have four calcium ions bound per mole protein. In the presence of 5 mM Mg^{2+} and 0.1 mM EGTA there should be no Ca^{2+} bound by calmodulin and binding sites with the ability to bind Mg^{2+} with a $k_d \leq 5.10\text{-}4M$ should be ∿90% occupied with Mg^{2+}.

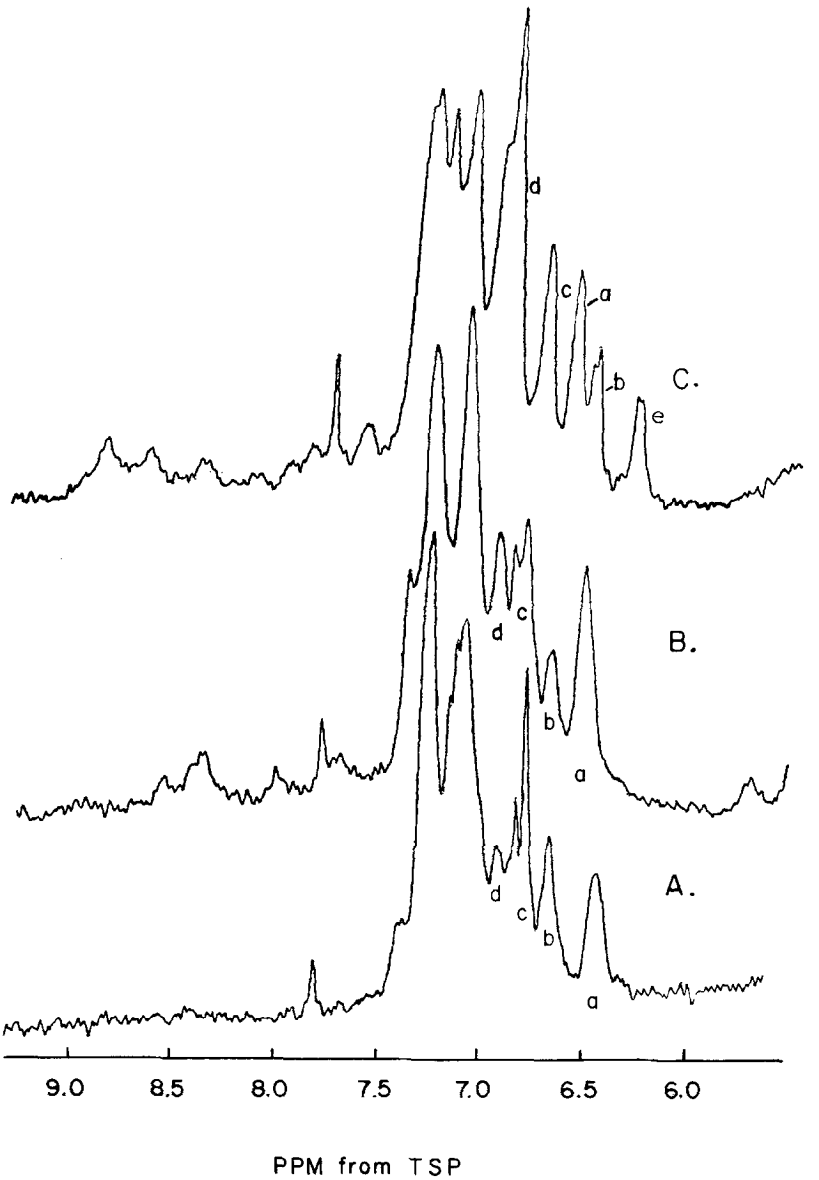

Figure 1.

Aromatic region of the spectrum of calmodulin; [calmodulin] ≃ 1 x 10^{-3}M, 0.2 M KCl, pH 7.5. (A) Ca^{2+}, Mg^{2+}-free; (B) [Mg^{2+}]free = 10 mM, Ca^{2+}-free; (C) [Ca^{2+}]free = 0.2 mM, Mg^{2+}-free. Resonance peaks discussed in Results are labeled (a-e).

the ring protons of the 8 phenylalanines, 2 tyrosines and single histidine of calmodulin. Two large peaks appear at 7.25 ppm and 7.09 ppm and are most

likely due to phenylalanine ring protons and meta protons of tyrosine. The single sharp peak at 7.80 ppm is assigned to the H-2 histidine proton. The resonance peaks between 6.25 ppm and 7.00 ppm are due to the ortho protons of the tyrosine's, the H-4 histidine proton, and any chemically shifted phenylalanine protons. The spectrum of calmodulin in the metal-free state is not indicative of a denatured or random coil conformation. The spectrum of the denatured protein would exhibit only one large peak for the phenylalanines, two smaller peaks for the ortho and meta protons of the two tyrosines, and two single peaks for the histidine H-2 and H-4 proton.

The spectrum of the aliphatic region of metal-free calmodulin (Figure 2A) is also not indicative of a denatured protein due to the appearance of ring current shifted aliphatic resonances in the region between 0.5 ppm and -0.2 ppm (labeled a-f in Figure 2A). These are characteristic of close interactions between aliphatic protons and aromatic rings which would not exist in a denatured protein. The large singlet peak at 3.08 ppm, is assigned to the methyl protons of the single trimethyllysine residue of calmodulin.

The spectra of calmodulin in the presence of Ca^{2+} (Figure 1C, 2C) or Mg^{2+} (Figure 1B, 2B) are also not indicative of a denatured protein; they both show ring current shifted resonances in the aliphatic region and many distinguishable resonances in the aromatic region. It is also apparent that there are distinct spectral differences between the spectra of Mg^{2+}-saturated and Ca^{2+}-saturated calmodulin. The aliphatic resonance at -0.19 ppm in the spectra of the metal-free protein (peak a, Figure 2A) does not appear in either the spectrum of the Ca^{2+} or Mg^{2+}-saturated protein; while the aliphatic resonance at 0.06 ppm (peak b, Figure 2A) appears shifted to 0.02 ppm in the spectrum of Mg^{2+}-saturated calmodulin and does not appear in the spectrum of Ca^{2+}-saturated calmodulin. Other spectral differences between the three conformations are seen in the regions between 1 ppm and 2.5 ppm which are due to side chain methylene and methionine methyl groups. The peak at 3.08 ppm assigned to the methyl group of the single trimethyllysine residue does not appear to be affected by metal binding.

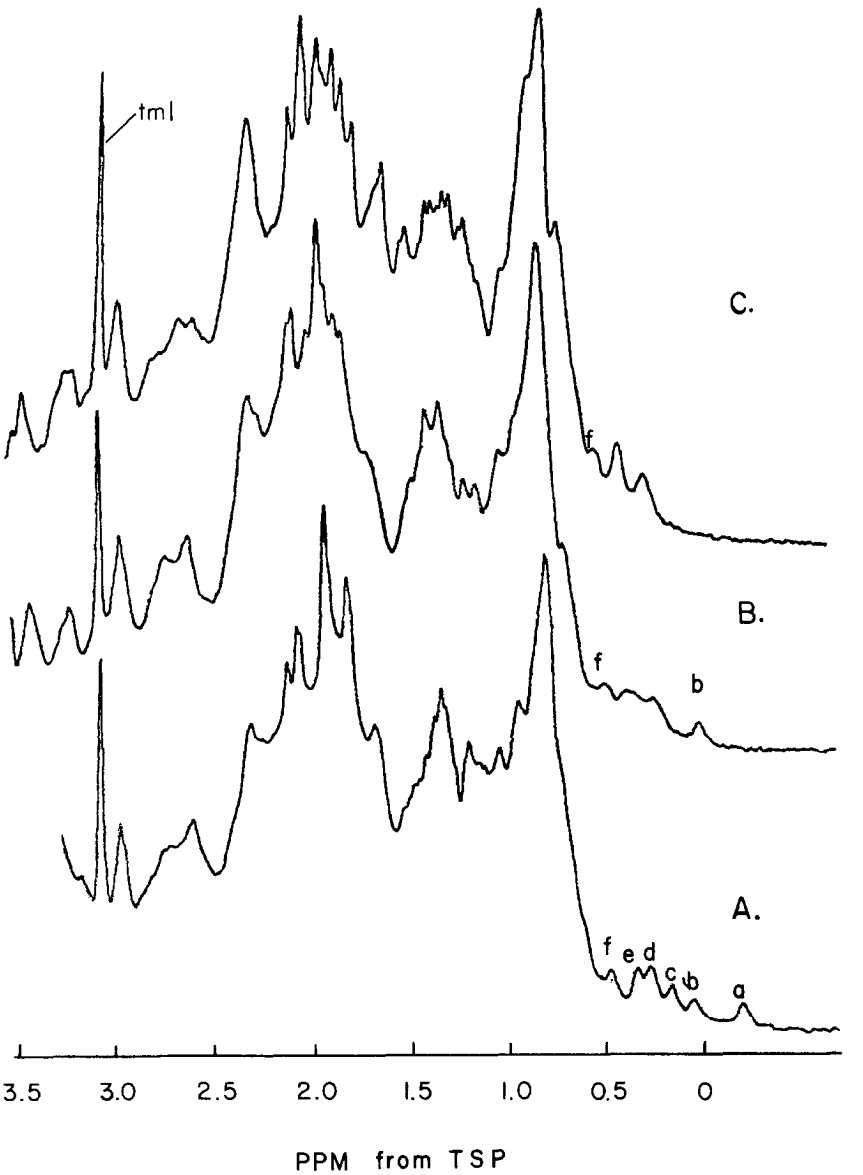

Figure 2.

Aliphatic region of the spectrum of calmodulin; [calmodulin] ≃ 1 x 10^{-3}M, 0.2 M KCl, pH 7.5. (A) Ca^{2+}, Mg^{2+}-free; (B) [Mg^{2+}]free = 10 mM, Ca^{2+}-free; (C) [Ca^{2+}]free = 0.2 mM, Mg^{2+}-free. Resonance peaks discussed in Results are labeled (a-f). The peak assigned to the methyl groups of the single trimethyllysine residue of calmodulin is labeled (tml).

In order to interpret the changes in the aromatic region, resonance assignments were made by titrating the protein in the alkaline pH range.

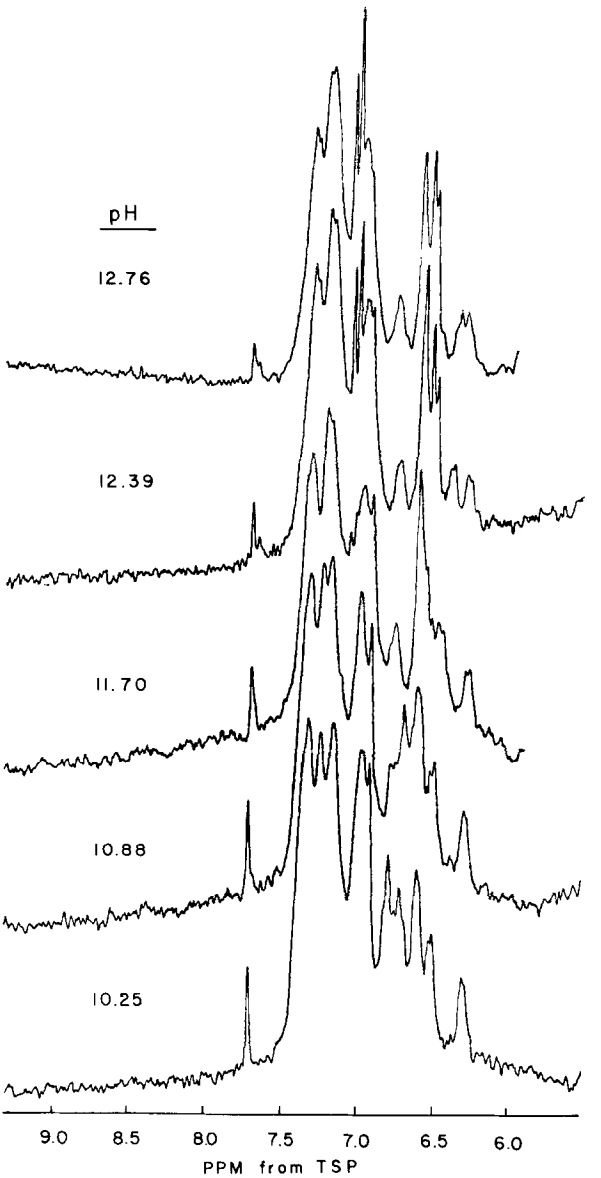

Figure 3.

Aromatic region of the spectrum of calmodulin as a function of pH; [calmodulin] ≈ 1 x 10-3M, 0.2 M KCl, pH 7.5. pH values are uncorrected for deuterium isotope effect and are indicated to the left of each spectrum.

Resonances due to tyrosine rings will shift upfield as the phenolic group is ionized. The spectra of calmodulin in the presence of Ca^{2+} at various pH values are shown in Figure 3. The peak at 6.73 ppm in the pH 10.25 spectrum

titrates upfield with an apparent pK of 10.4. (This peak corresponds to peak c in Figure 1C.) The peak appears as a doublet of about 8 hz coupling at 6.52 ppm in the pH = 12.39 spectra. Titrating in parallel with this peak is the doublet appearing at 7.04 ppm in the pH = 12.39 spectra. The parallel titration behavior and the 8 hz coupling of the two peaks suggests that they represent the ortho and meta protons of one of the tyrosines of calmodulin. Consistent with this interpretation is the report that tyrosine 99 of calmodulin has a pK of 10.4 in the Ca^{2+}-saturated conformation (30). The peak at 6.67 ppm in the spectrum of metal-free calmodulin (peak c, Figure 1A) also shifts upfield with an apparent pK of 10.3. It can be shown that this peak corresponds to the peak at 6.74 ppm (peak c, Figure 1C) in the spectrum of Ca^{2+}-saturated calmodulin. The difference in the chemical shift of this peak in the spectrum of Ca^{2+}-saturated calmodulin and in the spectrum of metal-free calmodulin suggests that the ring protons of this tyrosine are only slightly perturbed by metal binding. The peak at 6.52 ppm in the pH = 10.25 spectrum (corresponding to peak b in Figure 1C) does not shift until pH > 11.3 where it starts to shift upfield appearing at 6.34 ppm at pH = 12.76. The peak at 6.67 ppm in the metal-free spectrum (peak b, Figure 1A) also titrates upfield only at pH > 11.3. A Ca^{2+} titration of metal-free calmodulin indicates that this is the same peak appearing at 6.52 ppm (peak b, Figure 1C) in the Ca^{2+}-saturated spectrum (unpublished results). The chemical shift of this peak however does not appear to be affected by Mg^{2+} binding. This peak could be due to one of the ring protons of tyrosine 138 (reported to have a pK of 11.9 [30]); however, the assignment of this resonance will have to await studies on chemically modified calmodulin.

Specific aromatic resonances appear to be quite sensitive to the binding of metal ions. In particular, the peak at 6.44 ppm (peak a, Figure 1A) in the metal-free spectrum shifts downfield to 6.54 ppm in the spectrum of Mg^{2+}-saturated calmodulin. A Ca^{2+} titration indicated that as Ca^{2+} is added to metal-free calmodulin the peak shifts to 6.62 ppm (peak a, Figure 1C). This

shift appears to be representative of a Ca^{2+}-specific conformational change. Another spectral characteristic of the binding of metals to calmodulin is the increase in phenylalanine resonance intensity at 6.95 ppm (peak d, Figure 1A-C). There is a small increase in intensity at 6.95 ppm in the spectrum of Mg^{2+}-saturated calmodulin as compared to the spectrum of the metal-free protein; however, this peak is much larger in the spectrum of the Ca^{2+}-saturated protein, again characteristic of a Ca^{2+}-specific conformational change. The peak at 6.30 ppm in the spectrum of Ca^{2+}-saturated calmodulin (peak e, Figure 1C) also appears to be representative of a Ca^{2+}-specific conformation.

DISCUSSION

The NMR results indicate that calmodulin in the metal-free state has a definite tertiary structure. This is indicated by the presence of chemically shifted phenylalanine resonances and ring current shifted aliphatic resonances in the spectrum of the metal-free protein. The binding of Ca^{2+} or Mg^{2+} by calmodulin causes changes in the protein's conformation as indicated by specific spectral changes. Binding of Ca^{2+} or Mg^{2+} by metal-free calmodulin causes a decrease in intensity of the main phenylalanine peak at 7.25 ppm with an increase in intensity 0.3 ppm upfield. This metal ion induced upfield shift in phenylalanine intensity has also been observed in the spectrum of skeletal troponin-C upon addition of Mg^{2+} or Ca^{2+} to the metal-free protein (25-27). The Ca^{2+} dependence of the chemical shifts of certain upfield-shifted aliphatic resonances in the spectrum of metal-free calmodulin has also been observed for two upfield-shifted aliphatic resonances in the spectrum of metal-free troponin-C (25). Upfield-shifted phenylalanine resonances in the spectrum of troponin-C have been observed to shift downfield as a result of Ca^{2+} binding (25). This is similar to the behavior of aromatic resonance "a" in the spectrum of metal-free calmodulin.

The spectral changes caused by addition of Mg^{2+} or Ca^{2+} to metal-free calmodulin are qualitatively similar but show quantitative differences. This is most evident in the aromatic region where there is a very large increase in

phenylalanine intensity at 6.95 ppm upon addition of Ca^{2+}, while Mg^{2+} is only able to elicit a small increase. Further differences were indicated by Ca^{2+}-specific induced shifts of peaks in the aromatic region, and the differences in the upfield shifted aliphatic region between the spectra of Mg^{2+}-saturated calmodulin and Ca^{2+}-saturated calmodulin (see Results). If Ca^{2+} and Mg^{2+} can bind at the same sites then the spectral differences noted between the spectra of Ca^{2+} and Mg^{2+}-saturated calmodulin must be due to differences in the binding site geometry depending on which cation is occupying the site. It is also possible that Mg^{2+} is not able to bind at all of the four binding sites under the experimental conditions used in taking the NMR spectra (0.2 M KCl, pH 7.5), since it has been reported that high ionic strength can decrease the interaction of Mg^{2+} with testes calmodulin (5).

These results indicate that the binding of metals to skeletal troponin-C and brain calmodulin results in some similar conformational changes involving aromatic and aliphatic groups. However a comparison between the spectra of calmodulin and published spectra of skeletal troponin-C (25-27) shows that even though similar conformational transitions occur upon metal binding the overall conformations of the proteins are different. These differences and the concentration dependence of the metal ion induced conformational changes of calmodulin are being investigated.

ACKNOWLEDGEMENTS

This work was supported in part by USPHS research fellowship 1-F32-NS 05832-01 to K. Seamon and NIH grant RR 00292 to the Mellon Institute NMR Facility for Biomedical Studies. I would like to thank Krishna Bose for her assistance in taking the NMR spectra and Dr. Mordecai Blaustein for the use of his atomic absorption spectrophotometer. Some of this work was carried out in the laboratory of Dr. Blake Moore whose help and support is appreciated.

REFERENCES

1. Cheung, W.Y. (1970) Biochem. Biophys. Res. Comm. 38, 533-538.
2. Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974) J. Biol. Chem. 249, 4943-4954.
3. Wolff, D.J. and Brostrom, C.O. (1974) Arch. Biochem. Biophys. 163, 349-358.
4. Teo, T.S., Wang, T.H. and Wang, J.G. (1973) J. Biol. Chem. 248, 588-595.
5. Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977) J. Biol. Chem. 252, 8415-8422.
6. Waisman, D.M., Stevens, F.C. and Wang, J.H. (1978) J. Biol. Chem. 253, 1106-1113.

7. Childers, S.R. and Siegel, F.L. (1975) Biochem. Biophys. Acta 405, 99-108.
8. Kuo, I.C.Y. and Coffee, C.J. (1976) J. Biol. Chem. 251, 1603-1609.
9. Brostrom, C.O., Huang, Y.C., Breckenridge, B.M.L. and Wolff, D.J. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 64-68.
10. Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253-258.
11. Jarrett, H.W. and Pennsiton, J.T. (1978) J. Biol. Chem. 253, 4676-4682.
12. Schulman, H. and Greengard, P. (1978) Nature 271, 478-479.
13. Teshima, Y. and Kakiuchi, S.C. (1974) Biochem. Biophys. Res. Comm. 56, 489-495.
14. Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1976) Biochem. Biophys. Res. Comm. 68, 616-625.
15. Vanaman, T.C., Sharief, F. and Watterson, D.M. (1977) Calcium Binding Proteins and Calcium Function (Wasserman, R.H. et al., eds.) p. 107, Elsevier, Amsterdam.
16. Klee, C.B. (1977) Biochemistry 16, 1017-1024.
17. Wang, J.H., Teo, T.S., Ho, H.C. and Stevens, F.C. (1975) Adv. Cyclic Nucleotide Res. 5, 179-194.
18. Wolff, D.J., Poirier, P.G., Bromstrom, C.O. and Bromstrom, M.A. (1977) J. Biol. Chem. 252, 4108-4177.
19. Walsh, M. and Stevens, F.C. (1977) Biochemistry 16, 2742-2749.
20. Kuo, I.C.Y. and Coffee, C.J. (1976) J. Biol. Chem. 251, 6315-6319.
21. Lehrer, S.S and Leavis, P C. (1974) Biochem. Biophys Res. Comm. 58, 159-165.
22. Hincke, M.T., McCubbin, W.D. and Kay, C.M. (1978) Can J. Biochem. 56, 384-395.
23. Potter, J.D., Seidel, J.C., Leavis, P.C., Lehrer, S.S. and Gergely, J. (1976) J. Biol. Chem. 251, 7551-7556.
24. Head, J.F. and Perry, S.V. (1974) Biochem. J. 137, 145-154.
25. Seamon, K.B., Hartshorne, D.J. and Bothner-By, A.A. (1977) Biochemistry 16, 4039-4046.
26. Levine, B.A., Mercola, D., Coffman, D. and Thornton, J.M. (1977) J. Mol. Biol. 115, 743-760.
27. Levine, B.A., Thornton, J.M., Fernandes, R., Kelly, C.M. and Mercola, D. (1978) Biochem. Biophys. Acta 535, 11-24.
28. Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501-4513.
29. Dadok, J. and Sprecher, R.F. (1974) J. Magn. Reson. 13, 243-248.
30. Richmond, P.G. and Klee, C.B. (1978) Biochemistry 17, 928-935.