Comparison of Calcium-Modulated Proteins from Vertebrate Brains†

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ABSTRACT: Calmodulins have been purified from porcine, rabbit, rat, and chicken brains and their structural and functional properties compared to those of the bovine brain protein whose complete amino acid sequence has been elucidated. No major differences were detected in the amino acid compositions and tryptic peptide maps of these five proteins. All calmodulins lacked tryptophan and cysteine and contained 1 mol of N^{ϵ}-trimethyllysine and histidine per mol of protein. Bovine, porcine, rabbit, rat, and chicken brain calmodulins comigrated on polyacrylamide gels run under a variety of conditions in the presence and absence of denaturants. All brain calmodulins gave identical profiles for the calcium-dependent activation of "activatable" bovine brain 3′,5′-cyclic nucleotide phosphodiesterase. In addition, they formed calcium-dependent complexes with rabbit skeletal muscle troponin I and the electrophoretic mobilities of the complexes were identical with one another and similar to the corresponding complex between troponin I and troponin C. These studies more fully define what is a calmodulin, demonstrate that calmodulin is a relatively invariant constituent of vertebrate brain, and indicate that calmodulin structure and function have been highly conserved throughout vertebrate evolution.

An extensive body of evidence has accumulated demonstrating the importance of calcium in regulating the response of eucaryotic cells to external stimuli [for review, see Berridge (1975)]. Calcium appears to exert its biological effects as a second messenger or a signal transducer through its interaction with calcium-modulated proteins [for a review, see Kretsinger (1979)]. Recent studies have shown that a generalized calcium "target" protein, named calmodulin, exists in eucaryotic cells and regulates a number of enzyme systems in response to calcium flux [for review, see Kretsinger (1979)]. The enzymes regulated by calmodulin include a 3′,5′-cyclic nucleotide phosphodiesterase (Lin et al., 1974; Teo et al., 1973), an adenylyl cyclase (Cheung et al., 1975; Brostrom et al., 1975), a myosin light-chain kinase (Dabrowska et al., 1978; Hathaway & Adelstein, 1979), a membrane ATPase (Jarrett & Penniston, 1978; Larsen & Vincenzi, 1979), a phosphorylase kinase (Cohen et al., 1978), and an NAD kinase (Anderson & Cormier, 1978; Muto & Miyachi, 1977).

While it is generally agreed that calmodulins are acidic, heat-stable proteins which bind calcium with high affinity, the detailed properties reported (Brooks & Siegel, 1973; Dedman et al., 1977; Klee, 1977; Lin et al., 1974; Muto & Miyachi, 1977, Stevens et al., 1976; Teo et al., 1973; Wolff & Siegel, 1972; Wolff & Brostrom, 1974; Wolff et al., 1977) for calmodulin preparations used in reconstitution studies or isolated from enzyme preparations have been quite variable. Brain calmodulins have variously been reported to be phosphoproteins (Brooks & Siegel, 1973; Wolff & Siegel, 1972; Wolff & Brostrom, 1974) as well as lacking phosphate (Lin et al., 1974; Teo et al., 1973; Watterson et al., 1976; Wolff et al., 1977), to have blocked NH_2 termini (Dedman et al., 1977; Watterson et al., 1976, 1980) as well as to have free NH_2 termini (Lin et al., 1974), and to have varying physical and calcium binding properties (Dedman et al., 1977; Lin et al., 1974; Teo et al., 1973; Watterson et al., 1976; Wolff et al., 1972, 1977; Wolff & Siegel, 1972).

The reason for the apparently contradictory data in the literature is not clear. At least four possibilities exist: (1) calmodulins actually vary greatly in structure among vertebrate species, (2) more than one polypeptide possesses the ability to stimulate phosphodiesterase in a calcium-dependent manner, (3) a set of calmodulin-like proteins copurify because of their extremely similar chemical and physical properties and the relative proportions of these proteins in a purified preparation might vary as a function of the method of isolation, or (4) the method of isolation might result in alterations in the physical and chemical properties of the protein.

Published information concerning possible structural variation among calmodulins is far too incomplete at present to conclude that all calmodulins do not vary in structure. To date, complete amino acid sequence analysis has been reported only for bovine brain calmodulin (Vanaman et al., 1977; Watterson et al., 1980) and partial amino acid sequence analyses have been reported for calmodulins from bovine uterus (Grand & Perry, 1978) and rat testes (Dedman et al., 1978). The sequences of the bovine proteins are in agreement but appear to differ from the partial sequence of the rat testes protein. As part of a systematic study of the structure, function, and evolution of calmodulin, we have isolated calmodulin from bovine, porcine, rabbit, rat, and chicken brains by using the same isolation protocol for each species. As previously noted (Watterson et al., 1976), this isolation protocol is based on the physical, chemical, and calcium binding properties of calmodulin and results in the isolation of a unique chemical entity without using thermal denaturation steps or organic solvents and denaturants. We report here a comparison of the physicochemical, structural, and functional properties of the brain calmodulins from these four mammalian and one avian species. These studies more fully define what constitutes a calmodulin and demonstrate that the structural and functional properties of calmodulin from a single tissue have been even more highly conserved during vertebrate evolution than suggested by data in the literature. A preliminary report of this work has been presented (Vanaman et al., 1976).

Experimental Procedures

All chemicals were reagent grade and were utilized without further purification except as previously noted (Watterson et al., 1976). Skeletal muscle troponin I and troponin C were purified essentially as described by Cole & Perry (1975).

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Calmodulin was prepared from bovine, porcine, rabbit, rat, and chicken brains by using a modification of a previously described procedure (Watterson et al., 1976); after homogenization, only steps 4–6 were used to obtain homogeneous calmodulin. Partially purified phosphodiesterase was prepared from bovine brain, and phosphodiesterase activity was assayed as previously described (Watterson et al., 1976). Details of assays and contents of reaction mixtures are given where appropriate in the text and in figure legends.

Discontinuous polyacrylamide gel electrophoresis was performed as previously described (Watterson et al., 1976). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed exactly as described for discontinuous gel electrophoresis except sodium dodecyl sulfate was included in the upper and lower reservoir buffers and in the gel mix at a final concentration of 0.1% (w/v). Alkaline urea gels were run essentially as described by Head & Perry (1974). Slab gels consisting of 8% (w/v) acrylamide and 0.2% (w/v) bis-(acrylamide) contained 7.3 M urea (deionized), 0.015 M Trizma, 0.100 M glycine, adjusted to pH 8.5, 0.025% (w/v) ammonium persulfate, and 0.25% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. Gels were prerun with bromphenol blue tracking dye. Samples were applied, and electrophoresis was performed at 30 mA for 1.5–2 h. Both buffer chambers contained 0.015 M Tris and 0.100 M glycine, pH 8.5.

Trypsin digests of performic acid oxidized proteins were prepared exactly as previously described for bovine brain calmodulin (Watterson et al., 1976). Dried digests (0.5 mg) were dissolved in 50% (v/v) acetic acid and applied to Whatman 3MM chromatography paper. Descending chromatography was performed with pyridine–butanol–acetic acid–water (PBAW, 10:15:3:12 v/v/v/v) for 17 h at room temperature. After air-drying, high-voltage electrophoresis was performed at 90 °C in the second dimension by using a Savant flat plate electrophoresis apparatus with pyridine–acetic acid–water (10:100:2890 v/v/v) pH 3.5 buffer with 1% (w/v) methyl green as a marker. The papers were again air-dried, and peptides and amino acids were detected by staining with 0.5% (w/v) ninhydrin–0.1% (w/v) cadmium acetate reagent. As ninhydrin-positive spots appeared, major (i.e., intensely staining) spots were outlined with a solid line and minor (i.e., weakly staining) spots were outlined with dashed lines. The resultant "maps" were then directly photographed.

Ultraviolet absorption spectra were obtained with a Beckman Acta V recording spectrophotometer. Protein samples were hydrolyzed, and composition analyses were performed exactly as previously described (Watterson et al., 1976). Analyses of N^{ϵ}-trimethyllysine were performed on a column (0.9 × 55 cm) of Beckman Resin AA-15 by using the standard Beckman single-column methodology for acid hydrolysates at 55 °C.

Results

Physicochemical Properties of Purified Calmodulins. The methods described under Experimental Procedures for the isolation of calmodulins from brain homogenates utilize the unique size and charge properties of these proteins for purification. In addition, putative calmodulins are detected in fractions during purification by a gel electrophoretic method which depends on molecular size and surface charge. Bovine, procine, rabbit, rat, and chicken brain calmodulins showed identical mobilities on the 12.5% acrylamide gels used for assay purposes and identical characteristics at all stages of purification, indicating that they possessed similar charge and size properties. This was directly demonstrated by analyses of the purified brain calmodulins on (1) 8% acrylamide gels containing 8 M urea and run in a Tris–glycine buffer system (Figure 1, left panel) and (2) 15% acrylamide gels run in the presence of sodium dodecyl sulfate (Figure 1, right panel). Identical electrophoretic mobilities were observed for all calmodulins tested.

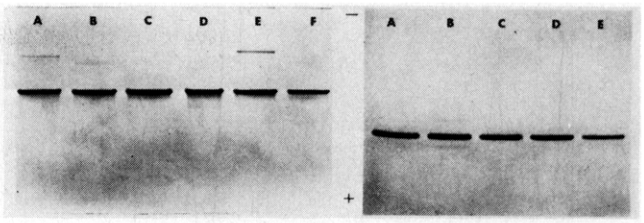

FIGURE 1: Electrophoretic analysis of brain calmodulins. Left panel: 8% polyacrylamide–alkaline urea gel. Samples were prepared in 5 M urea and 5 mM Tris–20 mM glycine, pH 8.5. Electrophoresis was performed as described under Experimental Procedures. The slots were loaded with 10 μg of (A) rabbit skeletal muscle troponin C and (B–F) bovine, porcine, rabbit, rat, and chicken brain calmodulins. Right panel: 15% polyacrylamide slab gel in 0.1% sodium dodecyl sulfate containing buffers. Samples were prepared and electrophoresis was performed as described under Experimental Procedures. The slots contained 10 μg of calmodulins from (A) bovine brain, (B) porcine brain, (C) rabbit brain, (D) rat brain, and (E) chicken brain. Direction of migration was as indicated for both separations.

The UV absorption spectra of porcine, rabbit, rat, and chicken brain calmodulins (Figure 2) are identical with that previously published for the bovine brain protein (Watterson et al., 1976) showing the characteristic fine spectrum of phenylalanine (Mihalyi, 1970). Spectra were obtained with protein solutions whose concentrations were determined by amino acid analysis. The slight variations observed in absorbancy at 276 nm and the depth of the trough at 255 nm in each spectrum were the result of slight contamination by nonprotein material as previously discussed (Watterson et al., 1976). When anomalously high extinction coefficients were obtained and no impurities were detectable by polyacrylamide gel electrophoresis, the calmodulin sample was taken through an additional ammonium sulfate precipitation at pH 4.2 and the resultant pellet was redissolved in a small volume of deionized water and desalted on a column of Sephadex G-10 in 0.01 M ammonium bicarbonate. After this treatment, the molar extinction coefficients at 276 nm for bovine, porcine, rabbit, rat, and chicken calmodulins were not significantly different from that previously reported (Watterson et al., 1976) for the bovine brain protein ($\epsilon_{M,276nm}$ = 3240). This value agrees with 2 mol of tyrosine per 17 000 g of protein and precludes the presence of tryptophan. The UV absorption spectrum of the porcine brain calmodulin shown here is identical with that published by Klee (1977) for the porcine brain activator protein. The $E_{276nm,1mg/mL}$ for these calmodulins calculated from the data in Figure 2 ranged from 0.16 to 0.20.

The amino acid compositions of these purified brain calmodulins are shown in Table I. All calmodulins were found to contain 1 residue of N^{ϵ}-trimethyllysine and 1 residue of histidine per 17 000 g. N^{ϵ}-Monomethyllysine, N^{ϵ}-dimethyllysine, and the methylhistidines were absent. Cysteine also was not present in any of the calmodulins, and cysteic acid was not detected in analyses of performic acid oxidized samples. Tryptophan was absent as judged by the UV absorption spectrum of each pure calmodulin (vide supra). The amino acid compositions given in Table I do not show any *major* differences from that of the bovine brain protein.

The inability to detect differences among these vertebrate brain calmodulins by gel electrophoretic methods, spectra studies, or amino acid analyses indicates that they have highly conserved amino acid sequences. However, it is clearly possible

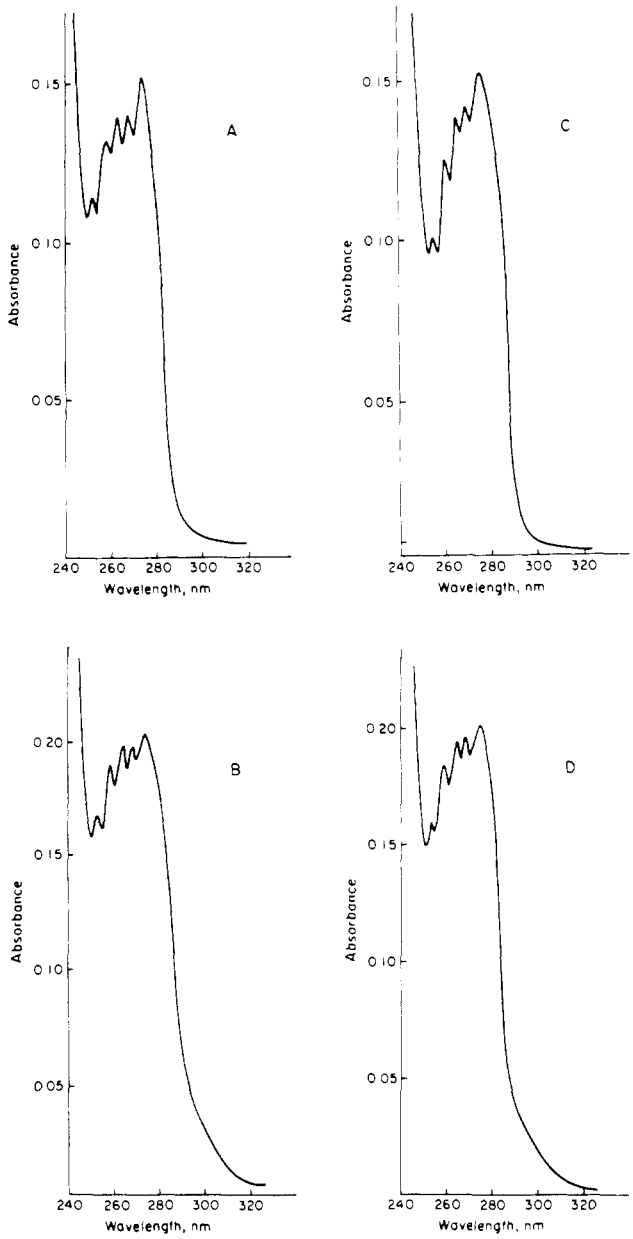

FIGURE 2: Brain calmodulin UV absorption spectra. Analyses were performed as described under Experimental Procedures. The spectra shown are from (A) porcine, (B) rabbit, (C) rat, and (D) chicken brain calmodulins.

for proteins to be related by amino acid composition yet still have unique amino acid sequences. The structural relatedness of these proteins was further examined by tryptic peptide mapping as described below.

Tryptic Peptide Maps. Extremely similar patterns were obtained when trypsin digests of bovine, porcine, rabbit, rat, and chicken brain calmodulins were analyzed by two-dimensional peptide mapping techniques as shown in Figure 3. While the resultant maps were not exactly superimposable, the *pattern* of major ninhydrin-positive spots was indistinguishable. The differences in mobility of analogous peptides from the various digests were so minor that detailed sequence analyses will be required in order to demonstrate the presence of any true amino acid sequence differences.

There were 14 major ninhydrin-positive spots observed in each map. This number of peptides is that expected from the lysine and arginine content of the respective proteins. However, studies of the performic acid oxidized bovine brain protein

Table I: Amino Acid Compositions of Brain Calmodulins

	mol/17 000 g					
	rabbit[a]	rat[a]	porcine[a]	chicken[a]	bovine[b]	bovine by sequence[b]
Lys	6.8	6.6	6.6	6.8	7.2	7
TML[c]	1.0	1.1	1.1	1.0	1.0	1
His	1.4	1.4	1.3	0.9	1.3	1
Arg	6.0	5.8	5.6	5.9	6.2	6
Asp	22.6	25.7	25.4	23.9	22.2	23
Thr	11.6	12.0	10.6	11.3	11.0	12
Ser	4.9	4.6	4.5	4.8	4.7	4
Glu	28.4	28.6	28.6	28.0	26.7	27
Pro	1.9	2.1	2.1	2.2	2.0	2
Gly	10.9	11.9	11.6	11.6	10.8	11
Ala	10.9	10.7	11.5	11.6	10.7	11
Cys	0	0	0	0	0	0
Val	7.4	7.4	6.9	7.3	7.2	7
Met	7.5	8.9	7.0	7.7	9.0	9
Ile	7.6	7.9	6.7	7.1	7.6	8
Leu	9.4	8.6	9.1	8.9	9.4	9
Tyr	2.1	2.1	2.4	2.3	2.0	2
Phe	7.8	8.2	7.7	7.8	7.8	8
Trp	0	0	0	0	0	0

[a] Determined as described in the text. [b] Values as reported elsewhere (Vanaman et al., 1977; Watterson et al., 1980). [c] TML = trimethyllysine.

(Watterson et al., 1980) have shown that (1) a number of lysine-containing peptide bonds are poorly cleaved by trypsin, (2) the trimethylated lysyl residue is totally resistant to hydrolysis, and (3) trypsin cleaves both methionylmethionine peptide bonds (residues 71 and 72; residues 144 and 145) in the unoxidized protein and this cleavage is not completely eliminated by their conversion to methionine sulfones. Therefore, it is not known if these major ninhydrin-positive spots are due *only* to cleavage at *all* available lysyl or arginyl residues in each calmodulin.

Phosphodiesterase Stimulatory Activity. The ability of calmodulins to stimulate bovine brain phosphodiesterase has been the major functional criterion used to test for specific calmodulin activity in most studies. However, the activation curves obtained in different laboratories have varied drastically depending on assay conditions, methods for quantifying calmodulin concentration, and the level of purity of different phosphodiesterase preparations. Therefore, it was necessary to compare directly the calcium-dependent activation activities of bovine, porcine, rabbit, rat, and chicken brain calmodulins in assays performed with the same stock of enzyme solution by using identical reaction conditions. Dose–response curves were determined for each calmodulin based on assays, performed in triplicate, at six different concentrations of added calmodulin varying from 0 to 4 μg/0.5 mL of reaction mixture. Calmodulin concentrations in stock solutions were determined by amino acid analysis. By use of phosphodiesterase (50 μg of protein per 0.5-mL reaction mixture) prepared and assayed with cAMP (2 mM) as a substrate as previously described (Watterson et al., 1976), all vertebrate brain calmodulins gave indistinguishable activation curves with 0.2–0.3 μg of added calmodulin required to give 50% of the maximum 5.0–5.5-fold activation. Fully activated phosphodiesterase had an activity of 0.20 μmol of cAMP hydrolyzed per min per mg of phosphodiesterase.

Formation of Ca^{2+}-Dependent Complexes with Rabbit Skeletal Muscle Troponin I. Amphlett et al. (1976) have previously reported that bovine brain calmodulin forms a calcium-dependent complex with rabbit skeletal muscle troponin I which is quite similar to the corresponding troponin

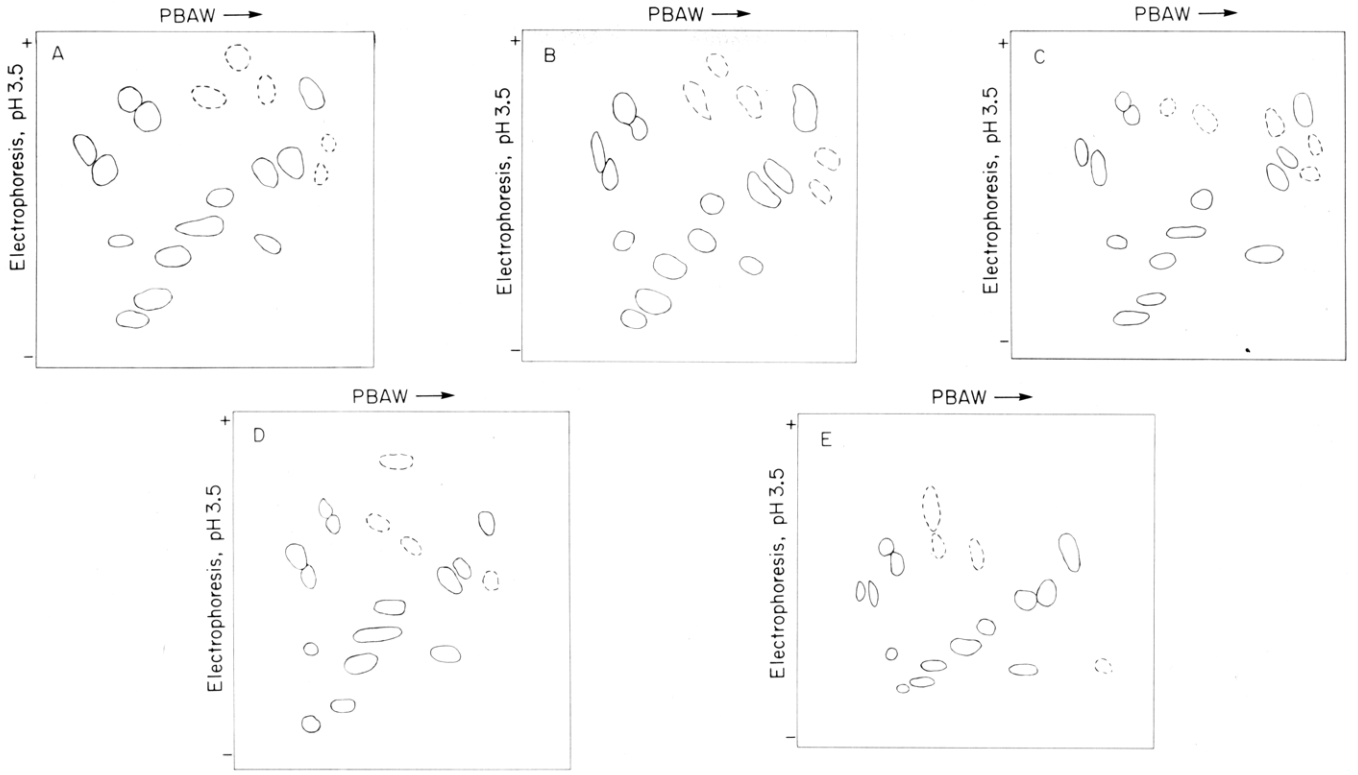

FIGURE 3: Tryptic peptide maps of brain calmodulins. Two-dimensional separations of trypsin digests of performic acid oxidized brain calmodulins were performed as described under Experimental Procedures. The intense (major) and weak (minor) ninhydrin-reactive spots were directly circled with solid or broken lines, respectively. These patterns were reproduced photographically without alteration. Samples analyzed were (A) bovine, (B) porcine, (C) rabbit, (D) rat, and (E) chicken brain calmodulins.

C–troponin I complex. Figure 4 shows that porcine, rabbit, rat, and chicken brain calmodulins and troponin I form calcium-dependent complexes whose electrophoretic mobilities are identical with the complex obtained with the bovine brain protein and troponin I. The calmodulin–troponin I complexes (slots H–L) all have reproducibly slower mobilities in the gel system used here than the homologous troponin I–troponin C complex (slot G). The presence of calmodulin–troponin I complex on the gel required calcium; inclusion of excess EGTA[1] in the samples (slots B–F) abolished the Coomassie blue staining band assumed to be complex (indicated by the arrows in the figure). The troponin I–troponin C complex (slot A) was only partially dissociated under these conditions (in the absence of urea). These studies demonstrate that those structural features required for troponin C like activity are present in the mammalian and avian brain calmodulins.

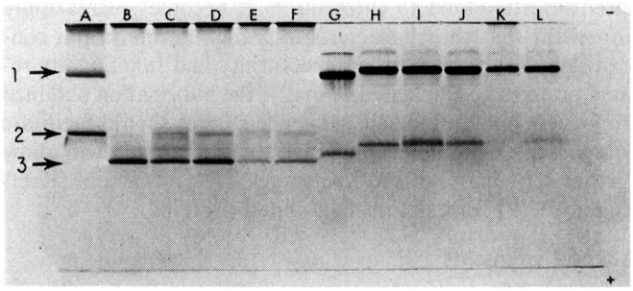

FIGURE 4: Demonstration of Ca^{2+}-dependent complex formation with rabbit skeletal muscle troponin I. Samples contained 10 μg of troponin I and 10 μg of troponin C or calmodulin in 50 μL of 10 mM Tris, 10 mM glycine, 2 mM 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue, pH 8.9. Samples in slots A–F contained 1 mM EGTA. Samples in slots G–L contained 1 mM $CaCl_2$. Prepared samples were analyzed immediately by electrophoresis on a 12.5% polyacrylamide slab gel with discontinuous buffers performed exactly as previously described (Watterson et al., 1976) except that EDTA was omitted from gel and reservoir buffers. Slots A and G contained rabbit skeletal muscle troponin C. Samples containing brain calmodulins were the following: (B and H) bovine; (C and I) porcine; (D and J) rabbit; (E and K) rat; (F and L) chicken. Arrow 1 denotes the mobility of complexes, arrow 2 the mobility of troponin C, and arrow 3 the mobility of calmodulin.

Discussion

The studies described in this report were undertaken to demonstrate in a systematic way that the physicochemical and functional properties of vertebrate calmodulins are invariant. Although there is general agreement in reports from different laboratories concerning the *gross* similarities among vertebrate calmodulins, considerable differences exist in the detailed physicochemical properties published for these proteins. The analyses reported here directly demonstrate that the structural and functional properties of calmodulin from a single tissue have been highly conserved during vertebrate evolution and exclude the possibility that vertebrate brain calmodulins vary significantly in structural properties. The detailed physicochemical properties reported here are in exact agreement with the data of Klee (1977) on porcine brain calmodulin but differ at least to some extent from previous reports (Brooks & Siegel, 1973; Dedman et al., 1977; Lin et al., 1974; Teo et al., 1973; Wolff & Siegel, 1972; Wolff & Brostrom, 1974; Wolff et al., 1977) on the characterization of several vertebrate calmodulins.

Detailed structural studies have been reported for only three calmodulins, those from bovine brain (Vanaman et al., 1977; Watterson et al., 1980), bovine uterine smooth muscle (Grand & Perry, 1978), and rat testis (Dedman et al., 1978). Apparent minor differences reported for assignments among these

[1] Abbreviations used: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

sequences have not been unequivocally proven and may be artifactual. The amino acid sequence of calmodulin from the invertebrate *Renilla reniformis* appears to differ from that of the bovine brain protein by only seven largely conservative replacements (Vanaman & Sharief, 1979). These differences can be detected by peptide mapping and amino acid compositional analysis. The identity in these properties noted here for vertebrate calmodulins suggests that they differ less in structure as a group than vertebrate compared to invertebrate calmodulins. Clearly, the structures of calmodulins do not vary significantly throughout the animal kingdom.

Demonstration of the invariant structure of vertebrate calmodulins is particularly important considering the wide variety of regulatory functions ascribed to it. The fact that other proteins such as brain S-100 (Watterson et al., 1976), striated muscle troponin C (Cohen et al., 1978; Yazawa & Yagi, 1977), and smooth muscle leiotonin C (Mikawa et al., 1978) with physicochemical and/or functional properties similar to calmodulin coexist with it in the same tissues is clearly a major problem since these proteins are difficult to resolve during purification. In addition, artifactual alterations in calmodulin properties have now been shown to result from various relatively mild conditions of treatment and storage (Jarrett & Penniston, 1978; Van Eldik & Watterson, 1979). The physicochemical properties described here for vertebrate calmodulins can be used as at least one major set of criteria for assaying putative homogeneous calmodulin samples.

In conclusion, these studies more fully define what is a calmodulin and demonstrate that calmodulin structure and function are more highly conserved than previously suspected from reports in the literature. Because of the multiple in vitro activities attributed to calmodulin, it becomes increasingly important that the characteristics which define it and contradistinguish it from other structurally and functionally related proteins be elucidated. Finally, the information obtained from these and amino acid sequence studies should facilitate comparative crystallographic investigations which will allow further refinement of the Kretsinger EF hand model (Kretsinger, 1979) for calcium-modulated proteins.

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