Structural Similarities between the Ca$^{2+}$-dependent Regulatory Proteins of 3':5'-Cyclic Nucleotide Phosphodiesterase and Actomyosin ATPase*

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Results of studies of the Ca$^{2+}$-dependent protein modulator of 3':5'-cyclic nucleotide phosphodiesterase isolated from bovine brain are presented which show its structural similarity to the Ca$^{2+}$-binding subunit of muscle troponin. Both proteins have blocked NH$_4$ termini, similar and characteristic ultraviolet absorption spectra, similar Ca$^{2+}$-binding properties, very similar amino acid compositions, and co-migrate on sodium dodecyl sulfate-polyacrylamide gels. The primary structures of selected tryptic peptides isolated from bovine brain modulator protein are similar or identical with regions of the primary sequences of rabbit skeletal muscle and bovine cardiac muscle troponin C. Bovine brain modulator protein contains an unidentified ninhydrin-positive basic compound not found in muscle troponin C. An improved procedure is presented which yields 40 to 70 mg of modulator protein per kg of bovine brain.

The divalent cation, Ca$^{2+}$, is involved in the regulation of numerous physiological and biochemical processes in animal tissues including such diverse processes as muscle contraction (1), microtubule assembly (2, 3), stimulus-secretion coupling (4), and the regulation of several enzyme activities. Because of its apparent role as a generalized regulatory signal, Rasmussen (5) has suggested that, like cyclic nucleotides, Ca$^{2+}$ should be afforded second messenger status.

Recent studies have indicated that Ca$^{2+}$ may play an extremely important role in regulating cyclic nucleotide metabolism. The most conclusive of these studies to date has involved the activation of cyclic nucleotide phosphodiesterase (EC 3.4.1.17) by Ca$^{2+}$. Kakinchi et al. (6) and Cheung (7, 8) independently demonstrated the presence of a factor in brain homogenates which, in the presence of Ca$^{2+}$, stimulated the activity of a brain cyclic nucleotide phosphodiesterase. This factor was subsequently shown to be a heat-stable phosphodiesterase activator protein that was present in high concentrations in numerous vertebrate neurosecretory tissues (9). This protein will be referred to as the modulator protein in this paper. Modulator protein activity has also been found in high concentrations in homogenates of a number of invertebrates.

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The abbreviations used are: TN-C, the calcium binding subunit of troponin; Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PTH, phenylthiohydantoin; EGTA, ethylene glycol bis-$\beta$-aminoethyl ether-$N,N'$-tetraacetic acid.
homogenate of a number of vertebrate brains. The TN C-like protein from bovine brain has been shown to be identical with bovine brain (this communication) and bovine heart (14) modulator protein. Studies presented in this paper provide further evidence for the structural similarities between muscle TN-C and bovine brain modulator protein. We also report that bovine brain modulator protein contains approximately 1 mol of an unidentified ninhydrin-positive compound/mol of protein. A new procedure for the purification of modulator protein from bovine brain is presented which gives better yields than that previously described (12).

EXPERIMENTAL PROCEDURES

Materials

Sequential grade dancyl chloride (10% solution in acetone), dancyl-amino acid standards, sodium dodecyl sulfate, t-norterucine, o-amino-o-

Beta-guanidinopropionic acid hydrochloride, ethanethiol, phenylisothio-
cyanate, dimethylthionamine, heptamethylenemine, hexamethylene-
chloride, ninhydrin, and hydroxindan were products of Pierce Chemi-
cal Co. Glass-distilled benzene, ethylacetate, and 1-chlorobutane,
obtained from Burdick and Jackson Laboratories, were used exclu-
sively as reagents. N,N'-Methylhistidine, N,N'-methylhistidine, and N'-monomethyllysine were used as supplied by Cycle Chemical
Co. L. Norleucine, L-norleucine, o-amino-
bis (2-nitrobenzoic acid), adenosine 3':5'-cyclic
phosphate, 5'.nucleotidase (Sigma grade II) and adenosine were
obtained from Eastman Organic Chemical. Ampholine carrier
gels were supplied by L. Levine, Brandeis University. Rabbit skele-
tal muscle TN-C was a gift of Dr. S. V. Perry (University of Birmingham,
England).

Methods

Preparation of Modulator Protein from Brain

Modulator protein was prepared using the procedure described
below. All operations were performed at 4°.

Step 1: Homogenization—Brains, obtained fresh from the slaughter-
house or from exsanguinated animals, were washed in physiological
NaCl solution, stripped of membranes and collagenous material, and
the spinal cord removed at the base of the brain. They were cut into
sections and stored frozen at 20° in 500-gram batches until used.

Frozen brains obtained from Pel Freeze Biological, Inc. (Rogers, Ark.) were thawed, washed in physiological saline, and cut into sections after removal of spinal cord and cerebellum.

One kilogram of tissue, thawed at 4°, was homogenized in 2-volumes of 0.3 M sodium acetate, 0.001 M 2-mercaptoethanol, and 0.001 M EDTA, pH 7.2 (Buffer A), at low speed in a Waring Blender for 90 s.

The homogenate was centrifuged at 10,000 rpm in a GSA rotor for 1 hour. The supernatant fluid was decanted and saved. The pellet was homogenized in an equal volume of buffer, centrifuged again, and the resulting supernatant fluid added to the original supernatant fraction. The pellets were saved.

Step 2: CM-Sephadex C-50 Batch Filtration—The combined super-
natant fractions were mixed with 800 ml of CM-Sephadex C-50
(packed volume) equilibrated with Buffer A. This mixture was allowed to stand for 30 min; then the resin was removed by filtration on a sintered-glass funnel. The packed resin bed was washed with 2 bed volumes of Buffer A and the washings added to the resin.

Step 3: First DEAE-Sephadex Column Chromatography—The en-
tire eluate from Step 2 was applied to a column (4 x 30 cm) of DEAE-Sephadex A-50 equilibrated with Buffer A. The column was washed with 300 ml of Buffer A followed by 300 ml of Buffer A containing 0.05 M NaCl. Adsorbed proteins were eluted with a linear salt gradient formed with 1 liter each of 0.05 M and 0.80 M NaCl in Buffer A as the starting and limit buffers, respectively. Ten-milliliter fractions were collected and monitored by reading absorbance at 235 nm. Aliquots of fractions in the region of absorbance peaks were analyzed on 12.5% discontinuous acrylamide gels (see below) and those fractions containing modulator protein were pooled.

Step 4: Ammonium Sulfate, pH 4.0, Precipitation—Solid ammo-
nium sulfate (313 g/liter) was added to the pooled fractions from Step 3 to bring the solution to 50% saturation. The resulting solution was adjusted to pH 7.0 with 1 N NH₄OH, stirred for 1 hour at 4°, then centrifuged for 30 min at 10,000 x g. The pellet was discarded. The supernatant fraction was adjusted to pH 4.0 with 1 N sulfuric acid to 50% ammonium sulfate. The mixture was stirred for 1 hour, centri-

guged at 10,000 x g for 30 min and the supernatant fluid discarded.

The pellet was resuspended in 10 to 40 ml of 0.01 M ammonium bicarbonate and dissolved by adjusting the slurry to pH 7.0 with 1 M Tris base. The protein was desalted on a column (4.0 x 45 cm) of Sephadex G-25 (coarse) in 0.01 M ammonium bicarbonate, shell-

from, and lyophilized.

Step 5: Second DEAE-Sephadex Column Chromatography—The lyophilized protein was dissolved in 50 ml of 0.01 M Tris-HCl, 0.001 M EDTA, 0.001 M 2-mercaptoethanol, and 0.2 M NaCl, pH 7.5 (Buffer B). The resulting solution was adjusted to pH 7.5 with 1 M Tris base and dialyzed against Buffer B. The dialyzed solution was clarified by centrifugation at 10,000 x g for 30 min and then applied to a column (4 x 40 cm) of DEAE-Sephadex A-50 equilibrated with Buffer B. The column was eluted with a linear salt gradient consisting of 1 liter each of Buffer B as starting buffer and Buffer C containing 0.7 M NaCl as a limit buffer. The modulator protein was located by reading absorbance at 235 nm, then analyzing appropriate fractions by 12.5% discontinu-
ous gel electrophoresis. Fractions were pooled and treated exactly as described in Step 4.

Step 6: Gel Filtration—The lyophilized protein from Step 5 was dissolved in 20 ml of 0.05 M Tris-HCl, 0.001 M EDTA, and 0.001 M 2-mercaptoethanol, pH 7.5, and applied to a column (5.4 x 133 cm) of Sephadex G-100 (fine) in the same buffer. The column was developed at 50 ml/hr. 10-milliliter fractions were collected and monitored as described above. The appropriate fractions were pooled, desalted, and lyophilized.

Preparations of Activator Deficient Enzyme

Partially purified phosphodiesterase was prepared from bovine brain using a modification of the procedure of Cheung (8). Bovine brain was homogenized and the homogenate treated exactly as described for Step 1 of the modulator protein purification scheme described above. The resultant supernatant was adjusted to 30% saturation by the addition of solid ammonium sulfate (0.47 g/liter). After standing for 1 hour at 4°, the suspension was centrifuged at 10,000 x g. The supernatant fluid was decanted and adjusted to 55% saturation by the addition of solid ammonium sulfate (0.47 g/liter). The pellet was collected as above, dissolved in a minimal amount of Buffer B, ex-

haustively dialyzed against deionized H₂O, shell-frozen, and lyophil-
ized. The lyophilized protein was dissolved in a minimal amount of Buffer B and the mixture subjected to chromatography as described in Step 5 of the modulator protein purification scheme, except that the linear salt gradient utilized Buffer B containing 0.5 M (NH₄)₂SO₄ as the limit buffer. The enzyme was located by reading absorbance at 280 nm, then analyzing appropriate fractions for phosphodiesterase activity. Fractions were pooled, dialyzed against deionized H₂O, and lyophi-
lized. The lyophilized solid was stored at -20°.

Assay of Phosphodiesterase Activator Activity

Phosphodiesterase activity and the ability of modulator protein to stimulate the activity of bovine brain cyclic nucleotide phosphodi-
esterase were measured as described for the previous section by using a modification of the procedure of Teo and Wang (11). Reaction mixtures of 0.3 ml contained 40 munits of Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM CaCl₂, 2 mM cyclic AMP, and an appropriate amount of enzyme and modulator protein. Production of 5'-AMP was measured by incubation with 5'-nucleotidase, after which inorganic phosphate was measured as described by Lowry (13).

Analytical Polyacrylamide Gel Electrophoresis—Analytical sodium
dodecyl sulfate-polyacrylamide electrophoresis was performed as previously described (20). Discontinuous polyacrylamide electrophoresis was performed using tube gels (0.6 x 12 cm) consisting of 12.5% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 4.5% (w/v) Tris, 0.06 M HCl, 0.0125% (w/v) N,N,N',N'-tetramethylmethylenediamine, and 0.75% (w/v) N,N',N'-tetramethylethylenediamine. The upper reservoir contained 0.25% (w/v) 2-mercaptoethanol. The lower reservoir contained 0.1 M Tris base/0.05 M HCl. Sample aliquots were dialyzed against a 1:5 dilution of upper reservoir buffer, mixed with an equal volume of 0.01% (w/v) bromophenol blue in 50% (w/v) glycerol, then applied to the gels. Electrophoresis was performed at 150 volts for 5 hours. Gels were stained with 0.25% (w/v) Coomassie brilliant blue R520 in 50% (v/v) methanol/10% (v/v) acetic acid for 1 hour at 37°, and destained for 2 hours in 7.5% (v/v) acetic acid and 5% (v/v) methanol at 37°.

**Gel Electrophoresis**—Isoelectric focusing gels were made with 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 6 M urea, 1.6% (w/v) LKB amphotolines of the desired pH range, 0.2% (w/v) N,N',N'-tetramethylmethylenediamine, and 0.12% (w/v) ammonium persulfate. The upper reservoir (cathode buffer) was 1% (w/v) ethylenediamine, and lower reservoir (anode vessel) buffer was 1.4% (v/v) orthophosphoric acid. Sample aliquots were contained in chambers of a microdialysis cell. Dialysis cells were sealed and equilibrated to equilibrium at 20,000 and 30,000 rpm in a Beckman model E ultracentrifuge equipped with an ultraviolet scanner. Polyacrylamide chain weight was determined by centrifugation of protein dissolved in 6 M guanidine hydrochloride. A partial specific volume of 0.72 cc/g was determined as the dansyl derivatives exactly as previously described (34).

**Phosphorus Content**—Phosphorus was determined as described by Peterson et al. (33). NH₂-terminal amino acids were determined as the dansyl derivatives exactly as previously described (34).

**RESULTS**

**Purification of Modulator Protein from Bovine Brain**—The modulator protein is a relatively small, acidic protein (11, 12) which migrates rapidly on high percentage polyacrylamide gels run with discontinuous buffer systems under electrophoresis conditions described under "Methods." We have used discontinuous electrophoresis exclusively for the detection and assay of this protein in crude homogenates of tissues and to monitor purification procedures. As shown in Table I, modulator protein represents approximately 1% of the soluble protein present in the initial bovine brain homogenate supernatant. While CM-Sephadex filtration (Step 2) gives only a 1.12 fold purification based on total protein, these proteins were detected and quantified in the various fractions obtained at each step in the purification by discontinuous electrophoresis. The amount of modulator protein present at each step was determined by comparison of the integrated areas of modulator protein peaks in the scans of gels containing known amounts of the purified protein to those obtained for known aliquots from each step.

As shown in Table I, modulator protein represents approximately 1% of the soluble protein present in the initial bovine brain homogenate supernatant. While CM-Sephadex filtration (Step 2) gives only a 1.12-fold purification based on total protein, this step removes residual particulate matter and lipid which would otherwise only be removed by high speed centrifugation (55,000 x g for 1 hour).
similar to those previously published for bovine brain (12) and heart (11) modulator proteins. The specific activity of the diesterase is shown in Fig. 5. This activator titration curve, obtained when sedimentation was performed in 0.01 M imidazole-HCl (gel B), or isoelectric focusing (gel A), while only one major band is detected when the same sample is subjected to sodium dodecyl sulfate-electrophoresis (see Fig. 4). These results suggest that the apparent contaminants seen in the original homogenate. The purity of this material was assessed by gel electrophoresis and electrofocusing. As shown in Fig. 4A, one major band and some minor, slower migrating bands are seen by nondenaturing discontinuous electrophoresis (gel A), while only one major band is detected when the same amount of protein is examined by discontinuous electrophoresis in the presence of 8 M urea (gel B), or isoelectric focusing in 6 M urea (gel C). A single band is seen when this sample is pooled fractions was recovered rapidly in high yield by precipitation with 50% ammonium sulfate, pH 4 (Step 4 in the procedure).

Table I

| Purification step | Total volume | Protein concentration | Total protein | Total modulator protein | Yield | Purification |
|-------------------|--------------|----------------------|---------------|-------------------------|-------|--------------|
| Step 1. Homogenization | 2,700 | 10.8 | 29,025 | 310 | 100 | 1.12 |
| Step 2. CM-Sephadex | 3,600 | 7.5 | 26,200 | 310 | 100 | 1.12 |
| Step 3. 1st DEAE-Sephadex | 980 | 5.3 | 3,470 | 243 | 78 | 6.5 |
| Step 4. 50% (NH₄)₂SO₄, pH 4.0 | 35 | 97.2 | 3,401 | 220 | 71 | 6.5 |
| Step 5. 2nd DEAE-Sephadex | 70 | 0.7 | 49 | 46 | 15 | 89.0 |
| Step 6. Gel filtration | 41* | 41* | 10 | 10 | 10 |

* Determined by the method of Lowery et al. (38).
* Determined by gel scanning as described in text.
* For percentage of yield, the amount of modulator protein present at Step 1 was assumed to be 100%. Fold purification was calculated as (total modulator protein/total protein) at each step divided by (total modulator protein/total protein) at Step 1.
* Not significant.
* Determined by amino acid analysis.

Fig. 2 shows the elution profile obtained with DEAE-Sephadex A-50 (Step 5). The third major peak, eluting at 0.5 M sodium chloride, contained modulator protein as shown by the gels in the inset. The peak eluting at 0.45 M sodium chloride is the acidic, brain-specific protein Fraction S-100.

Gel filtration of the modulator protein pool (Fractions 155 to 170) from Step 5 on Sephadex G-100 is shown in Fig. 3. The peak indicated as “pooled” contained pure brain modulator protein. A trace identical with that shown in Fig. 3 was obtained when the S-100 pool (Fractions 120 to 140) from Step 5 was chromatographed on the same Sephadex G-100 column. The overall yield of bovine brain modulator protein obtained with this procedure (~40 mg/kg of brain) is 13% of that present in the original homogenate. The purity of this material was assessed by gel electrophoresis and electrofocusing. As shown in Fig. 4, one major band and some minor, slower migrating bands are seen by nondenaturing discontinuous electrophoresis (gel A), while only one major band is detected when the same amount of protein is examined by discontinuous electrophoresis in the presence of 8 M urea (gel B), or isoelectric focusing in 6 M urea (gel C). A single band is seen when this sample is subjected to sodium dodecyl sulfate-electrophoresis (see Fig. 7). These results suggest that the apparent contaminants seen in Fig. 4A are aggregates of the modulator protein. The ability of bovine brain modulator protein prepared by the above procedures to stimulate activator-depleted phosphodiesterase is shown in Fig. 5. This activator titration curve is identical with that of purified bovine heart modulator protein. The polypeptide chain weight of modulator protein was determined by two methods. Equilibrium sedimentation of the purified bovine brain protein dissolved in 6 M guanidine hydrochloride gave the results shown in Fig. 6B. At equilibrium, an uncorrected molecular mass of 15,256 daltons was calculated from the slope of this plot. Sodium dodecyl sulfate-electrophoresis of purified bovine brain modulator protein and standard proteins of known molecular weight, shown in Fig. 7, gave an apparent chain mass of 18,000 ± 500 daltons in agreement with the values obtained by sedimentation equilibrium under nondenaturating conditions. In addition, bovine brain modulator protein co-migrated with rabbit muscle TN-C, an acidic protein whose chain mass is 17,923 daltons calculated from its amino acid sequence (40).

Amino Acid Composition The amino acid composition (moles of amino acid/18,000 g of protein) of the purified modulator protein shown in Table II was determined from duplicate analyses of 24-, 48-, 72-, 96-, and 120-hour hydrolysates. Threonine and serine values were corrected for destruc-
Similarities between Calcium-dependent Regulatory Proteins

FIG. 2. DEAE-Sephadex A-50 chromatography (Step 5). Chromatography was performed as described in the text with 10-ml fractions collected. Modulator protein (MP) and S-100 were detected in fractions by discontinuous polyacrylamide electrophoresis as shown by the gels positioned above the fractions examined. A pin was inserted in each gel at the position of the bromophenol blue tracking dye after electrophoresis. S-100 co-migrated with tracking dye in this system. Fractions 155 to 170 were pooled for modulator protein purification, while Fractions 129 to 140 were pooled for S-100 purification.

FIG. 3. Gel filtration on Sephadex G-100 (Step 6). Gel filtration was performed as described under "Methods." Ten milliliter fractions were collected as indicated on the figure and treated as described in the text.

FIG. 4. Electrophoresis and electrofocusing of purified bovine brain modulator protein. Gel A, 12.5% discontinuous electrophoresis, 50 μg of protein; Gel B, 12.5% discontinuous electrophoresis in the presence of 8 M urea, 50 μg of protein; Gel C, gel isoelectric focusing in 6 M urea, 50 μg of protein. Gels were run, stained, and destained as described under "Methods."

FIG. 5. Activation of activator-depleted cyclic nucleotide phosphodiesterase by purified bovine brain modulator protein. Assays were performed as described in the text using 0.5 ml of incubation mixtures containing 0.144 unit (micromoles/min/mg of protein) of phosphodiesterase, and varying amounts of modulator protein (MP) as shown. ●, the mean values for triplicate determinations; error bars indicate the standard deviation from the mean.

Compound X shown in Table II was originally detected in acid hydrolysates of a purified trypsinic peptide isolated from the bovine brain protein (see below). This basic, ninhydrin-positive compound elutes coincident with lysine and N'-monomethyllysine from the regular short column (0.9 x 10 cm) of the amino acid analyzer. The unknown compound also eluted coincident with N'-monomethyllysine when analyses were performed on a Beckman 121 M amino acid analyzer with a column (0.25 x 25 cm) of Spinco AA-20 resin. Under the conditions described under "Methods," lysine and ornithine eluted from this column at 73 min after injection followed by N'-monomethyllysine (81 min), histidine (90 min), and N\(^\prime\)-and N\(^\prime\)-methylhistidines (97 min).

Compound X co-chromatographed with N\(^\prime\)-methylhistidine...
Fig. 6. Molecular weight determination by sedimentation equilibrium. A, native molecular weight determinations were performed as described under "Methods" by sedimentation to equilibrium at the speeds shown. Protein concentration was 1.05 mg/ml. B, equilibrium sedimentation of bovine brain modulator protein (1.05 mg/ml) in 6 M guanidine hydrochloride was performed at a rotor speed of 40,000 rpm. The temperature was 25° in all runs.

Fig. 7. Sodium dodecyl sulfate-polyacrylamide electrophoresis analysis of bovine brain modulator protein. Purified proteins were disrupted in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 0.1 M sodium phosphate, pH 6.2, by boiling for 2 min. Samples were then applied to a slab gel consisting of 10% acrylamide/0.2% bisacrylamide containing 0.1% sodium dodecyl sulfate, 1 mM EDTA, 0.1 M sodium phosphate, and 8 M urea, pH 7.2. Electrophoresis was performed for 17 hours at 50 mA with 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 1 mM EDTA, and 0.1 M sodium phosphate, pH 7.2, as electrode buffer. A, bovine brain modulator protein, 15 µg. B, Rabbit skeletal muscle TN-C, 15 µg. C, molecular weight standards including Escherichia coli aspartate transcarbamylase, 5 µg; bovine α-lactalbumin, 5 µg; and bovine heart cytochrome c, 2 µg.

when analyzed using standard Beckman single column methodology as described under "Methods." Under these conditions, histidine eluted at 210.8 min after sample injection followed closely by N'-methylhistidine (212.3 min), N'-methyllysine (224.0 min), and ammonia (232.0 min). N'-diand trimethyllysines were not tested.

As shown in Table II, 24-hour hydrolysates of bovine brain modulator protein contained 1.1 mol of Compound X/18,000 g of protein calculated using the ninhydrin color constant for histidine. No such ninhydrin-positive compound was detected in hydrolysates of rabbit skeletal muscle TN-C, in agreement with the amino acid sequence previously published (40).

Analyses of acid hydrolysates of 1- to 2-mg quantities of purified bovine brain modulator protein for inorganic phosphorus show less than 1 mol of phosphorus/18,000 g of protein. Analyses for carbohydrate were negative.

The amino acid compositions of rabbit muscle TN-C, hake parvalbumin, and bovine brain S-100 are also shown in Table II. The amino acid compositions of bovine brain modulator protein and rabbit muscle TN-C are strikingly similar, as has also been noted for the bovine heart protein (13, 14). The only significant differences are (a) the presence of 12 residues of threonine in the modulator protein as compared to 7 in muscle TN-C; (b) the presence of a single residue of cysteine in rabbit muscle TN-C which is absent in the brain protein; and (c) the presence of an unknown ninhydrin-positive compound in the modulator protein and not in skeletal muscle TN-C. The amino acid composition of hake parvalbumin is quite distinct from either the brain or rabbit muscle protein, despite the fact that parvalbumin appears to share a common genetic ancestry with skeletal muscle TN-C (40). The amino acid composition of the S-100 protein fraction from bovine brain is clearly not related to brain modulator protein.

NH₂-terminal Determinations—No α-dansyl amino acids could be detected when 10 nmol of bovine brain modulator protein were subjected to procedures previously described for determination of free NH₂-terminal amino acids by the dansyl technique. When 250 nmol (4.52 mg) of this protein were subjected to automated Edman degradation in a Beckman model 890B Sequencer as described under "Methods," no PTH-derivatives were detected during 10 cycles of degradation. Sperm whale myoglobin applied to the cup immediately after the 10th cycle gave only the appropriate amino acids at each cycle for 10 cycles of degradation with an average repetitive yield of 92%.

These data suggest that, as with skeletal and cardiac muscle TN-C's (40, 42), bovine brain modulator protein has a blocked NH₂ terminus. The bovine heart protein also appears to have a blocked NH₂ terminus (13).

Spectral Properties—Fig. 8 shows the spectrum obtained with bovine brain modulator protein at neutral pH. This spectrum is striking due to the presence of the phenylalanine
Table II

Comparative amino acid compositions of muscle and brain calcium binding proteins

| Bovine brain modulator protein* | Assumed mol of amino acid per mol of protein | Rabbit skeletal muscle* troponin C | Hake* paravalbumin | Bovine S-100β |
|--------------------------------|---------------------------------------------|-----------------------------------|-------------------|---------------|
|                                | mol/18,000 g                               | residues/molecule                 |                   |               |
| Lysine                         | 8.9                                        | 9                                 | 12                | 18            |
| Histidine                      | 1.4                                        | 1                                 | 1                 | 8             |
| Compound X                     | 1.1                                        | 1                                 |                   |               |
| Arginine                       | 6.7                                        | 7                                 | 7                 | 1             |
| Aspartic acid                  | 23.9                                       | 24                                | 22                | 22            |
| Threonine                      | 11.8                                       | 12                                | 6                 | 6             |
| Serine                         | 5.1                                        | 5                                 | 7                 | 11            |
| Glutamic acid                  | 28.8                                       | 29                                | 31                | 10            |
| Proline                        | 2.2                                        | 2                                 | 1                 | 0             |
| Glycine                        | 11.7                                       | 12                                | 13                | 12            |
| Alanine                        | 11.5                                       | 12                                | 13                | 19            |
| Half-cystine                   | 0                                          | 1                                 | 1                 | 4             |
| Valine                         | 7.8                                        | 8                                 | 7                 | 15            |
| Methionine                     | 9.7                                        | 10                                | 10                | 1             |
| Isoleucine                     | 8.2                                        | 8                                 | 9                 | 7             |
| Leucine                        | 10.3                                       | 10                                | 9                 | 8             |
| Tyrosine                       | 2.2                                        | 2                                 | 2                 | 3             |
| Phenylalanine                  | 8.4                                        | 8                                 | 10                | 10            |
| Tryptophan                     | 0                                          | 0                                 | 0                 | 1             |

a Determined as described in the text.

b Based on the amino acid sequence published by Collins et al. (40).

c Taken from Pechere et al. (41).

d Taken from Dannies and Levine (18).

The molar extinction coefficient of the bovine brain protein determined by these spectral analyses was ε 276 nm = 3240 (E 1% 1 cm, 276 nm = 0.18). This agrees well with 2 mol of tyrosine/18,000 based on ε 276 nm = 1367 for tyrosine at neutral pH (44). As the molar extinction coefficient of tryptophan is 5430 at 276 nm (44) the presence of 1 mol of tryptophan/M, = 18,000 g of protein is unequivocally precluded.

Because of its spectral properties, empirically derived methods for determining protein concentration by spectral measurements such as that of Warburg and Christian (45) give anomalously low values for solutions of purified modulator protein.

Ca** Binding—Fig. 9 shows the results of equilibrium dialysis binding experiments with bovine brain modulator protein and "Ca**. The Scatchard plot of these data indicates that there are two sets of Ca**-binding sites. High affinity binding (Kd = 1 x 10^-4 M) of 2 mol of Ca** bound/18,000 g of protein was observed. Two additional mol of Ca**/18,000 g of protein bound with a dissociation constant of 8.6 x 10^-4 M. These data are very similar to those previously reported by Lin et al. (12) for the bovine brain protein and by Teo and Wang (11) for bovine heart modulator protein.

Tryptic Peptides of Bovine Brain Modulator Protein—The data presented in the preceding sections have shown that bovine brain modulator protein appears to be structurally related to rabbit skeletal muscle TN-C. Near identity in the amino acid sequences of two proteins can usually be demonstrated by two-dimensional tryptic peptide mapping techniques. However, proteins with closely related amino acid sequences (e.g., hen egg white lysozyme and bovine α-lactalbumin (46)) might give very dissimilar tryptic peptide maps. The comparative tryptic peptide maps of bovine brain and heart modulator proteins are clearly identical with one another and, with the exception of a single peptide, totally different from that obtained for skeletal muscle TN-C (13).
Since all modulator proteins tested to date appeared to have blocked NH₂ termini, comparison of their NH₂-terminal sequences to rabbit skeletal muscle TN-C by automated Edman degradation was not possible. Therefore, studies of the tryptic peptides isolated from bovine brain modulator protein were undertaken to provide evidence of structural similarity to rabbit skeletal muscle TN-C. Fig. 10 shows the elution profile obtained when a trypsin digest of the perimicro acid-oxidized protein was chromatographed on Beckman AA-15 as described in the figure legend. Thin layer chromatography of aliquants (5 to 10 nmol) of pooled fractions gave the results shown by circles on figure. Pool 1 was further resolved into three major peptides by gel filtration on Sephadex G-50 (fine) as shown in Fig. 11A. Thin layer chromatography of the fractions pooled as indicated in this figure showed that pools A and C contained pure peptides. Pool B gave a faint ninhydrin-positive spot which was subsequently detected with starch-iodide spray (47). No NH₂ terminus could be detected for this peptide by the dansyl method (see Table III).

The peptides produced by trypsin digestion of bovine brain modulator protein in the presence of 1 mM EGTA have also been purified exactly as described in the legend to Fig. 10. The elution profile (data not shown) obtained for these peptides was similar to that shown in Fig. 10 except that (a) peptide 4 was not resolved from the group of peaks labeled 5 in Fig. 10; and, (b) a new peptide (Tp-8A) was isolated eluting between peptides 8 and 9 in the profile shown in Fig. 10. The pooled fractions containing the unresolved peptide 4 from this separation were resolved into three pure peptides by gel filtration as shown in Fig. 11B.

The amino acid compositions and NH₂ termini of the pure tryptic peptides isolated from the bovine brain protein are presented in Table III. The values given were determined by analysis of 24-hour acid hydrolysates. The values in parentheses are assumed residues/molecule. The percentage of yield of each tryptic peptide based on micromoles of starting material are also given. Peptides Tp-5A, 5B, and 8A were obtained from the EGTA-trypsin digest of bovine brain modulator protein; the remainder were obtained from the perimicro acid-oxidized protein. Peptide Tp-5A was the only peptide isolated which contained either histidine or Compound X.

The amino acid composition of peptide Tp-4 corresponds to residues 38 through 44 in the sequence of rabbit skeletal muscle TN-C previously reported (40) and is identical with that of a tryptic peptide isolated from bovine heart modulator protein (13, 14).
Table III

Tryptic peptides of bovine brain modulatory protein

| Peptide | 1A | 1B | 1C | 2 | 3 | 4 | 5A | 5B | 6 | 7 | 8A | 9 | 10 | 11 |
|---------|----|----|----|---|---|---|----|----|---|---|----|---|----|----|
| Lysine  | 0.2| 1.0| 1.0| 0.2| 1.8| 0.2| 0.2| 1.0| 1.0| 1.0| 1.0| 1.0| 1.0| 2.0|
| Histidine| 0.5| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7|
| Compound.d | 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9|
| Arginine | 0.8| (1)| 0.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1|
| Aspartic Acid | 5.7| (6)| 0.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1|
| Threonine | 2.8| (3)| 0.3| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1|
| Serine  | 0.9| (1)| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5| 0.5|
| Glutamic Acid | 6.8| (7)| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6| 2.6|
| Proline | 2.1| (2)| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9|
| Glycine | 3.0| (5)| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0| 2.0|
| Alanine | 2.9| (3)| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4| 2.4|
| Valine | 1.1| (1)| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7| 1.7|
| Methionine.d | 0.7| (2)| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7| 0.7|
| Isoleucine | 0.9| (1)| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1|
| Leucine | 3.0| (3)| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9| 0.9|
| Tyrosine | 7.0| (1)| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1| 0.1|
| Phenylalanine | 1.9| (2)| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2| 1.2|
| Total assumed residues | 0.7| (1)| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1| 1.1|
| % Yield | 40 | 26 | 26 | 55 | 80 | 78 | 64 | 56 | 26 | 26 | 26 | 26 | 26 | 26 |
| ML.Terminus.d | Met | N/O | Thr | Asx | Glx | Glx | His | Val | Met | -- | Val | Thr | Glx | Met | Lys |

(a) Amino acid composition: determined as described in text; values in parentheses are assumed residues per mole.

(b) Purified from EGA trypsin digested bovine brain modulatory protein.

(c) Determined by single column methodology as described in METHODS.

(d) Determined as methionine sulfone except for peptides 5A, 5B, and 8A.

(e) Determined by the dansyl procedure; N/D = none detected.
Similarities between Calcium-dependent Regulatory Proteins

Table IV
Partial amino acid sequences of bovine modulator protein tryptic peptides

| Peptide | Amino Acid Sequence |
|---------|---------------------|
| Tp-2    | Asx - Thr - Asx - Ser - Glx - Glx - Glx - Ile - Arg |
| Tp-3    | Glx - Ala - Phe - Thr - Leu - Phe - Asx - Thr - Asx - Gly - Thr - Gly - (Asx, Ile, Ser, Lys) - Lys |
| Tp-4    | Glx - Leu - Gly - Thr - Val - Met - Arg |
| Tp-5B   | Val - Phe - Asx - Lys - Asx - Gly - Asx - Gly - Tyr - Ile - (Asx, Ala, Glx, Ser, Leu, Ala) - Arg |
| Tp-6    | Met - Lys - Asx - Thr - Asx - (Ser, Glx, Glx, Glx, Ile) - Arg |
| Tp-8    | Val - Phe - Asx - Lys |
| Tp-8A   | Thr - Ala - Lys |
| Tp-9    | Glx - Ala - Phe - Arg |
| Tp-10   | Met - Ala - Arg - Iys |
| Tp-11   | Lys - Met - Lys |

* - indicates determined by the dansyl Edman procedure as described under "Methods."

Table V
Alignment of modulator protein tryptic peptides and muscle TN-C amino acid sequence by apparent homology

| Peptide | Amino Acid Sequence |
|---------|---------------------|
| Tp-5    | Glx - Ala - Phe - Thr - Leu - Phe - Asx - Thr - Asx - Gly - Thr - Gly - (Asx, Ile, Ser, Lys) - Lys |
| Tp-6    | Met - Lys - Asx - Thr - Asx - (Ser, Glx, Glx, Glx, Ile) - Arg |
| Tp-8    | Val - Phe - Asx - Lys |
| Tp-8A   | Thr - Ala - Lys |
| Tp-9    | Glx - Ala - Phe - Arg |
| Tp-10   | Met - Ala - Arg - Iys |
| Tp-11   | Lys - Met - Lys |

Table V
Alignment of modulator protein tryptic peptides and muscle TN-C amino acid sequence by apparent homology

| Peptide | Amino Acid Sequence |
|---------|---------------------|
| Tp-5    | Glx - Ala - Phe - Thr - Leu - Phe - Asx - Thr - Asx - Gly - Thr - Gly - (Asx, Ile, Ser, Lys) - Lys |
| Tp-6    | Met - Lys - Asx - Thr - Asx - (Ser, Glx, Glx, Glx, Ile) - Arg |
| Tp-8    | Val - Phe - Asx - Lys |
| Tp-8A   | Thr - Ala - Lys |
| Tp-9    | Glx - Ala - Phe - Arg |
| Tp-10   | Met - Ala - Arg - Iys |
| Tp-11   | Lys - Met - Lys |

* - indicates determined by the dansyl Edman procedure as described under "Methods."

DISCUSSION

The results of purification and characterization of bovine brain modulator protein reported in this study differ in a number of ways from the results of Lin et al. (12). The purification procedure reported here appears to yield 10 times more material per kg of bovine brain than obtained by their procedure. The yield of pure modulator protein which they reported (4.2 mg/kg of brain) was measured by the method of Warburg and Christian (45), a spectrophotometric technique using the empirical relationship:

\[
\text{Protein concentration (mg/ml)} = 1.55 (A_{280nm}) - 0.67 (A_{260nm})
\]  

(1)

The modulator protein does not obey this empirical relationship, as noted under "Results." For example, the absorbances at 280 and 260 nm of 1 mg/ml solutions of the bovine brain protein would give a value of 0.17 mg/ml if substituted into Equation 1. This would indicate that Lin et al. (12) actually...
obtained approximately 25 mg of pure modulator protein/kg of brain with their purification procedure. This also reduces the specific activity of the pure protein substantially. Using a specific activity corrected by multiplying that reported by 0.17, the total amount of modulator protein in their brain homogenate would have been 948 mg/kg of tissue. This is very close to the value of 310 mg/kg which was obtained in our homogenate supernatant as judged by discontinuous electrophoresis (see Table I). Therefore, it is concluded that the assay of modulator protein by discontinuous electrophoresis is at least as accurate as that obtained by enzymic methods. In addition, it appears that the procedure described under “Methods” solubilizes bovine brain modulator protein efficiently. In contrast, brain homogenates prepared by the procedure of Lin et al. (12) in a buffer containing no chelating agents yielded only ½ of the total modulator protein in a soluble form, the remainder being lost in the insoluble fraction after centrifugation of the crude homogenate. The procedure described under “Methods” utilized buffers containing 1 mM EDTA in most steps including homogenization. Only trace amounts of modulator protein were detected by discontinuous electrophoresis of samples of the resuspended pellet obtained from the crude homogenate. This more efficient extraction during homogenization probably accounts for the higher yields of the pure protein obtained using the procedure described under “Methods.”

As noted under “Results,” one of the major problems encountered in the purification of modulator protein from brain is to separate it from the acidic S-100 protein fraction. In our procedure, this was accomplished by ion exchange chromatography on DEAE-Sephadex A-50 with a relatively shallow salt gradient (Step 5). Gel filtration on Sephadex G-100 is then sufficient to yield modulator protein which is more than 96% pure as judged by gel electrophoresis under a number of different conditions.

It is not possible to directly correlate the specific activity of the bovine brain modulator protein purified here with the values published by Lin et al. (12) because of differences in assay procedures. However, it does appear to have a specific activity identical with the bovine heart protein. *1

The procedure described in this paper has been used to prepare modulator protein from a number of sources including porcine, rabbit, rat, and chicken brains. *1 In all cases, 40 to 70 mg of protein were isolated/kg of brain. The purity of these preparations was comparable to that of the bovine brain modulator protein shown here. In large scale purification (2 to 6 kg of brain) a modification of the procedure described under “Methods” has been devised which yields 70 to 100 mg of modulator protein/kg of bovine brain.

Bovine brain modulator protein isolated by the procedures reported here is identical in physicochemical properties with that isolated and characterized by Lin et al. (12) with two important exceptions. It is reported here that this protein contains approximately 1 mol of an unknown ninhydrin-positive compound, Compound X/mol of protein. This compound which is not well resolved from lysine with the standard two column method of Moore and Stein (49) has been used for amino acid analysis in previous studies (11, 12) and used for amino acid analysis in previous studies (11, 12) has been localized in a single tryptic peptide isolated from bovine brain protein, peptide Tp-5A (see Table III). It also has been found in bovine heart modulator protein (13) and in modulator proteins prepared from porcine, rabbit, rat, and chicken brains. *1 The structure of Compound X is currently under investigation.

* No NH₂-terminal amino acid was detected in the current studies for the purified bovine brain modulator protein by either dansylation or automated Edman degradation. Similar observations have been made for the bovine heart protein (13). Using the dansyl method, Lin et al. (12) detected an NH₂-terminal valine in their preparations of bovine brain modulator protein. The reason for these differences is unclear. However, primary structure analyses currently in progress in our laboratory should provide precise information concerning the NH₂-terminus of this protein.

The other physicochemical properties of bovine brain modulator protein presented here are in general agreement with those determined by Lin et al. (12) and are identical with those of the bovine heart protein (13).

A number of Ca⁺⁺-binding proteins have been isolated from brain and other neurosecretory tissue. The S-100 protein fraction from brain, originally isolated by Moore and McGregor (49) and subsequently shown to be a calcium-binding protein (50) has been studied extensively. However, no biological function has yet been determined for S-100. In addition, the heterogeneity of this protein fraction (51-53) and the fact that physiological concentrations of monovalent cations abolish high affinity Ca⁺⁺ binding indicate that S-100 is not a unique, specific Ca⁺⁺-binding protein. The amino acid composition of bovine S-100 is clearly distinct from bovine brain modulator protein as can be seen in Table II. In addition, S-100, a number of other small acidic Ca⁺⁺-binding proteins have been isolated from brain. Wolff and Siegel (54) have reported the isolation and characterization of a Ca⁺⁺-binding phosphoprotein from porcine brain. Brooks and Siegel (55) have reported the isolation of a similar protein from bovine brain and adrenal medulla. In both cases, the molecular masses of these proteins were determined by sedimentation to be approximately 12,000 daltons. However, these analyses were performed in dilute buffers (0.01 M phosphate, pH 7.0), conditions which give anomalously low molecular weights for bovine brain modulator protein as noted under “Results.” The amino acid compositions of these brain and adrenal medulla proteins calculated using a molecular mass of 18,000 daltons are very similar to that of bovine brain modulator protein reported here. However, unlike these phosphoproteins which contain 2 mol of phosphorus/12,000 g of protein, bovine brain modulator protein prepared by our procedure contains no detectable phosphate, in agreement with the results of Lin et al. (12) and Teo and Wang (11) for bovine brain and heart proteins, respectively. The amino acid composition reported for the porcine brain phosphoprotein (54) is quite distinct from either the bovine brain and adrenal medulla phosphoproteins or bovine modulator protein.

Recent studies for the purified bovine brain modulator protein as judged by amino acid composition, NH₂-terminal analysis, and tryptic peptide mapping, and is devoid of phosphorus. *1

Recently, Wolff and Brostrom (56) and Brostrom et al. (57) have shown that the phosphoprotein isolated from bovine brain is an activator of cyclic nucleotide phosphodiesterase (57) and also appears to activate adenylate cyclase (57). Cheung et al. (58) have also shown that bovine brain modulator protein can activate adenylate cyclase and appears to be bound to detergent-solubilized adenylate cyclase preparations.
The physiological significance of the activation of both synthetic and degradative enzymes for 3':5'-cyclic AMP by a single regulatory protein is unclear at present.

As is noted under "Results," the physicochemical properties of bovine brain modulator protein are very similar to those of rabbit skeletal muscle TN-C, as has also been noted for bovine heart activator (13, 14). Comparative tryptic peptide maps of the modulator protein bear little resemblance to those of rabbit skeletal muscle TN-C. However, the amino acid sequences of tryptic peptides isolated from bovine brain modulator protein appear to be for the most part very similar to regions of the sequence of rabbit skeletal muscle TN-C published by Collins et al. (40) as shown in Table V. In the case of peptide Tp 4, the sequence is identical with a tryptic peptide expected from rabbit skeletal muscle TN-C following trypsin cleavage at lysine 37 and arginine 45. Kettsinger and Barry (59) have predicted a three-dimensional structure for rabbit skeletal muscle TN-C in which the side chains of numerous residues in this region of the linear sequence are involved in liganding Ca2+.

Although the complete primary structure of bovine brain modulator protein currently under investigation in our laboratory will be required to unequivocally prove that it is structurally related to muscle TN-C, the studies presented in this paper indicate strongly that such structural similarities exist. The presence in neurosecretory tissue of high concentrations of neurostenin system is intimately involved in mediating the effects of brain modulator protein currently under investigation in our laboratory.

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