Comparison of Calcium-binding Proteins

BOVINE HEART AND BRAIN PROTEIN ACTIVATORS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND RABBIT SKELETAL MUSCLE TROPO닌 C*

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In previous studies we have shown that the activation of bovine heart cyclic nucleotide phosphodiesterase by purified protein activator is completely dependent on the presence of Ca2+ and that the protein activator-Ca2+ complex is probably the true activator for the enzyme (Teo, T. S., and Wang, J. H. (1973) J. Biol. Chem. 248, 5930-5955). More recent studies have led us to believe that the mechanism of the Ca2+ activation of phosphodiesterase resembles that of the Ca2+ activation of muscle contraction and that the protein activator may play a role similar to troponin. In the present study we show that the protein activator resembles rabbit muscle troponin C in amino acid composition, molecular weight, isoelectric point, and ultraviolet absorption spectrum. Preliminary structural studies also indicate that these two proteins may have evolved from a common ancestral protein through gene duplication. This argument is strengthened by the finding that the tryptic peptide map of the bovine heart protein activator is indistinguishable from that of the bovine brain phosphodiesterase activator protein for which preliminary sequence information also suggests homology to troponin C (Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501-4513).

Cheung (3, 4) and Kakiuchi et al. (5) independently discovered the existence of a protein activator for cyclic nucleotide phosphodiesterase in rat brain; Goren and Rosen (6) found a similar protein to be present in bovine heart. Recently the activators from bovine heart (7) and bovine brain (8) have been extensively purified. Studies using the purified activator from either heart (7, 9) or brain (8) have shown that the activators are Ca2+-dependent and that their active form is Ca2+-activator complex. It has been postulated that the activator exists in an inactive conformation which may be converted to an active state upon binding of Ca2+. The increased resistance of the activator to proteolysis in the presence of Ca2+ (10) lends support to this postulate. The Ca2+-activator complex then associates with the phosphodiesterase to form a protein-protein complex with a biological activity severalfold that of the free enzyme (11, 12). We have previously suggested (1) that the mechanism of Ca2+ activation of phosphodiesterase resembles the mechanism of Ca2+ regulation of actomyosin ATPase which is also modulated by a specific Ca2+-binding protein, troponin (13, 14). Troponin contains three distinct polypeptide chains (15): the calcium-binding protein TN-C,† the inhibitory protein TN-I, and the tropomyosin-binding protein TN-T. TN-I exerts an inhibitory effect on actomyosin ATPase and the inhibition can be abolished by TN-C in the presence of calcium. This Ca2+ regulation is enhanced by TN-T which binds tropomyosin. Although the muscle system is more complex, the similarities in the modes of action of protein activator and TN-C are obvious. Troponin has a restricted distribution in the animal kingdom (16) and lower forms such as the Mollusca and Brachipoda do not contain this calcium-binding protein but our studies on the protein activator (17) suggest that it is ubiquitous in the animal kingdom.

In this report we compare the physical and chemical properties of bovine heart protein activator and TN-C and we provide some evidence for the postulate (1) that these two proteins have evolved from a common ancestor. While this work was in its final stages we became aware of the studies of Vanaman et al. (18) on a troponin C-like Ca2+-binding protein from brain; we therefore extended our comparison to include that protein, which has now been shown to be the phosphodiesterase activator from bovine brain.

EXPERIMENTAL PROCEDURES

Materials

Bovine heart protein activator of cyclic nucleotide phosphodiesterase was purified according to the procedure of Teo et al. (7) and

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†The abbreviations used are: TN-C, troponin C; TN-I, troponin I; TN-T, troponin T.
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analyzed by disc gel electrophoresis for homogeneity. All samples used were essentially pure unless indicated otherwise. Activator-deficient cyclic nucleotide phosphodiesterase was prepared from bovine heart as described previously (7). Samples of rabbit skeletal muscle TN-C were gifts from Dr. L. B. Smillie of the University of Alberta and Dr. J. T. Sturtevant of the University of California, Davis. Tropomin C-like Ca2+-binding protein from bovine brain was a gift from Dr. V. Vanaman of Duke University. Ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid was purchased from Sigma (St. Louis, Mo.) and N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin was obtained from Worthington Biochemicals (Freehold, N. J.). All other chemicals were reagent grade or better.

Methods

Assay Procedure—The activity of phosphodiesterase was measured by the procedure of Butler and Sutherland (19) as described in detail in a previous communication (7). The protein activator was assayed for its ability in activating a standard amount of activator-deficient phosphodiesterase (0.4 to 0.5 units). One unit of activator was defined as the amount giving 50% maximal activation of the enzyme. The possible activation of the enzyme by other Ca2+-binding proteins was analyzed in the same way.

Protein Determination—Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as the standard.

Analytical Ultracentrifugation—Analytical ultracentrifugation was carried out with a Beckman Spinco model E analytical ultracentrifuge. Sedimentation equilibrium runs were carried out at 20° and a rotor speed of 22,000 rpm. Both Rayleigh and schlieren optics were used. The buffer density was measured with a pycnometer. The partial specific volume, , of the protein sample was calculated from the amino acid composition of the protein using the partial specific volume of the constituent amino acids according to Cohn and Edsall (22).

Isoelectric Focusing—Isoelectric focusing experiments were carried out in a LKB isoelectric focusing column (model 8101) with a capacity of 110 ml. Protein samples (1 to 2 mg) were focused for 3 days at 4° using pH 3 to 10 ampholytes on a 0 to 40% sucrose gradient. Fractions of 1.5 ml each were then collected and analyzed.

Acrylamide Gel Electrophoresis—Disc gel electrophoresis was carried out according to the procedure of Davis (22) at pH 8.6. For most experiments both 12 and 15% gels were used. Samples were applied in 20 to 30% sucrose. The gels were stained with Amido black (1% in 7% acetic acid). Sodium dodecyl sulfate gel electrophoresis was carried out according to the procedure of Weber and Osborn (23) using 10% acrylamide gels. Protein samples were applied in 30% sucrose. Gels were stained with 2.5% Coomassie blue in 50% methanol and 9.2% acetic acid and destained in 7% acetic acid.

Amino Acid Analysis—Samples containing 0.02 to 0.1 μmol of reduced and alkylated protein were hydrolyzed with 6 N HCl (containing 2 μl of thiglycolic acid and 50 μl of 5% phenol/ml to protect carboxymethylcysteine and tyrosine against destruction (24) at 110° in sealed evacuated tubes for 24, 48, and 78 hours. The analyses were carried out on a Spinco 120/80 amino acid analyzer as outlined in the Spinco manual.

Reduction and Alkylation—The protein was dissolved in 0.1 M Tris-HCl, pH 8.5, containing 6 μg guanidine-HCl, 1 mg of EDTA/ml, and 0.01 M dithiothreitol to a concentration of 2 mg/ml. After 2 hours at room temperature an equal volume of 0.2 M Tris-HCl, pH 8.5, containing 0.05 M iodoacetic acid was added to the reaction mixture. After a further hour at room temperature all the material was applied to a column (1.5 × 30 cm) of Sephadex G-25; 10% acetic acid was used as the eluant. Fractions containing protein material were pooled and lyophilized.

Digestion with Trypsin and Peptide Mapping—A sample of protein was dissolved in 100 μl of 0.1 M ammonium bicarbonate—0.1 M ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid. The solution was saturated with nitrogen and 5 μl of N-tosyl-L-phenylalanine chloride was added, the tube was covered, mixed, and incubated at 37° for 2 hours. A sample (50 μl) of the digest was applied to Whatman No. 3MM paper and subjected to two-dimensional peptide mapping. High voltage electrophoresis was performed in a Savant electrophoresis tank at pH 4.7 as previously described (26) with Methyl green (1%) as a marker (26). Descending chromatography was carried out in the other dimension using butanol/pyridine/acetic acid/water (120/80/24/6, v/v) as the solvent. After drying, peptide spots were detected with the ninhydrin-collidine reagent (27).

In order to obtain enough peptide for amino acid analysis, the material obtained from 4 to 5 mg of protein was subjected to peptide mapping; the maps were stained with 0.01% ninhydrin in 85% ethanol at room temperature and the spots were cut out, washed in ether, and eluted first with 10% aqueous acetic acid and then with 50% aqueous pyridine. The material was taken to dryness in a vacuum desiccator, hydrolyzed in 6 N HCl, 110° for 24 hours, and subjected to amino acid analysis.

RESULTS

Physical Properties—Previous molecular weight determinations (7) for bovine heart protein activator by sedimentation diffusion and gel filtration techniques resulted in different values: 19,000 and 27,000, respectively. Similarly, Lin et al. (8) have recently determined the molecular weight of bovine brain protein activator and also obtained different values with different techniques: 15,000 and 28,000 with sedimentation equilibrium and gel filtration methods, respectively. In this study, a molecular weight of 16,800 has been obtained by sedimentation equilibrium. A partial specific volume, , of 0.72 ml/g was calculated from the amino acid composition of the protein and the protein sample (1.2 mg/ml in buffer containing 20 mM Tris, 1 mM magnesium acetate, 10 μM calcium chloride, and 0.1 M sodium chloride. pH 7.5) was centrifuged at 22,000 rpm for 25 hours at 20°.

On sodium dodecyl sulfate-gel electrophoresis, the bovine heart protein activator migrates as a single band with a mobility corresponding to a molecular weight of 18,000. This value is similar to the molecular weight as determined by ultracentrifugal methods (19,000 and 17,000), suggesting that the protein activator is a monomeric protein. The same conclusion was reached for bovine brain protein activator by Lin et al. (8). However, these investigators found a somewhat lower molecular weight, 15,000, for the brain activator on sodium dodecyl sulfate-gel electrophoresis.

Rabbit skeletal muscle TN-C was used as one of the markers for the molecular weight determination of bovine heart activator by sodium dodecyl sulfate-gel electrophoresis. It was found that the protein activator and TN-C had identical mobility. When mixtures of the two proteins were applied to the gel, only one protein band was detected. Thus, it is clear that the bovine heart protein activator and rabbit skeletal muscle TN-C have similar if not identical molecular weights. The molecular weight of rabbit skeletal muscle TN-C has been determined by many investigators and found to range from 17,000 to 22,000 (15, 28–30). From the amino acid sequence of TN-C, a molecular weight of 17,846 has been calculated (31). Although bovine activator and TN-C move together on sodium dodecyl sulfate-gel electrophoresis, they can be separated by polyacrylamide gel electrophoresis on 15% gels at pH 8.6 under nondenaturing conditions.

Figure 1 shows the isoelectric focusing profile for the bovine heart protein activator. The activator activity peak is similar if not identical molecular weights. The molecular weight determination of bovine heart protein activator by sodium dodecyl sulfate-gel electrophoresis was initiated by Lin et al. (8). Hartshorne and Dreizen (29) have carried out isoelectric focusing on rabbit skeletal muscle TN-C; purified TN-C was found to give rise to three proteins (32-35). Fig. 2 shows that the ultraviolet spectrum of bovine heart protein activator is atypical of common globular proteins (32-35).
the bovine heart protein activator resembles that of TN-C. Both spectra exhibit considerable vibrational structure in the region of 250 to 280 nm. Absorption peaks are seen at approximately 253, 259, 265, and 268 nm. The maxima of the spectra are in the region of 275 to 278. The absorbances in this region (ε253, 259, 265, 268, and 276 nm) may be calculated for the protein activator and TN-C and they are 1.9 and 2.1, respectively.

Amino Acid Composition—Table II gives the amino acid composition of reduced and alkylated bovine heart protein activator and compares it to those of rabbit skeletal muscle TN-C and also bovine heart TN-C. It is clear from these results that all three proteins are very similar in composition, with the activator possibly being more similar to rabbit skeletal muscle TN-C than to bovine heart TN-C. The proteins are characterized by a high content of acidic amino acids, absence of tryptophan, little or no cysteine, and a high phenylalanine to tyrosine ratio (see Table II).

Table I summarizes and compares some of the physical parameters of rabbit skeletal muscle TN-C and bovine heart protein activator.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protein activator</th>
<th>TN-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation constant (S)</td>
<td>2.0</td>
<td>1.92</td>
</tr>
<tr>
<td>Molecular weight:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analytical ultracentrifugation</td>
<td>17,000–19,000</td>
<td>22,000</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate-gel electrophoresis</td>
<td>18,000</td>
<td>18,000</td>
</tr>
<tr>
<td>pH (pH)</td>
<td>4.0</td>
<td>4.1, 4.4</td>
</tr>
<tr>
<td>Extinction coefficient (1% protein, 1 cm)</td>
<td>1.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* From Teo et al. (7).
† From Murray and Kay (30).
‡ From Hartshorne and Dreizen (29).
Vanaman (Duke University) and also found to contain approximately 1 residue of the same unidentified amino acid. In our routine acid hydrolysate methodology this modified amino acid would elute together with lysine. The composition of the bovine heart activator reported here is virtually identical with that reported by Lin et al. (8) for the protein activator isolated from bovine brain. The NH₂-terminus of the bovine heart activator is probably blocked since we were unable to degrade the protein by Edman degradation on a Beckman automatic sequenator.

Tryptic Peptide Maps—Fig. 3 shows the tryptic peptide maps of bovine heart protein activator, bovine brain troponin C-like calcium-binding protein (TCLP), and rabbit skeletal muscle TN-C. BPAW, butanol/pyridine/acetic acid/water (120/80/24/96, v/v).

Fig. 3. Tryptic peptide maps of bovine heart protein activator, bovine brain troponin C-like calcium-binding protein (TCLP), and rabbit skeletal muscle TN-C. BPAW, butanol/pyridine/acetic acid/water (120/80/24/96, v/v).
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The peptide maps of the protein activator and of troponin C-like calcium-binding protein have the same specific activity in stimulating activator deficient phosphodiesterase as does the bovine heart activator, has led us to the conclusion that these two proteins are probably identical. On the other hand a purified sample of rabbit skeletal muscle TN-C failed to activate the enzyme and the tryptic peptide map of rabbit skeletal muscle TN-C is surprisingly different from that of the other two proteins. All the major peptides from both the activator and TN-C maps were eluted and their amino acid composition determined as described under “Experimental Procedures.” Only the peptide indicated by the arrow on Fig. 3 has an identical amino acid composition in activator and TN-C; it corresponds to residues 38 to 44 in the TN-C sequence published by Collins et al. (31): -Glu-Leu-Gly-Thr-Val-Met-Arg-.

This peptide was found to have the same sequence in bovine brain tropolin C-like calcium-binding protein but the sequence in that region of bovine heart TN-C (37) is different in that the threonyl residue shown above is replaced by a lysine in bovine cardiac muscle TN-C.

DISCUSSION

The results from the present study and the accompanying paper by Watterson et al. (39) lead us to the conclusion that the phosphodiesterase activator proteins from bovine heart (7, 9) and bovine brain (8) and the troponin C-like calcium protein from bovine brain recently isolated by Vanaman et al. (18) are one and the same protein. The tryptic peptide maps of the phosphodiesterase activator protein from bovine heart and of the troponin C-like calcium-binding protein from bovine brain are indistinguishable and both proteins have the same specific activity with respect to their ability to stimulate cyclic nucleotide phosphodiesterase from bovine heart. As judged by peptide mapping both proteins yield the same 20 major peptides by trypsin digestion. From the amino acid composition one would, on the basis of 8 lysine residues and 6 arginine residues, predict only 15 tryptic peptides; it therefore appears that some nonspecific peptide bond hydrolysis has taken place during digestion with trypsin. This is not likely to be due to chymotrypsin contamination since N-tosyl-L-phenylalanine-chloromethyl ketone treated trypsin was used for these experiments.

We have previously postulated (1) that the protein activator of phosphodiesterase and muscle troponin-C may be homologous proteins evolved from a common ancestor. In comparing the tryptic peptide maps of the activator proteins from bovine heart and bovine brain on the one hand and rabbit skeletal muscle troponin-C on the other hand one would, at first, conclude that there is little or no homology between activator and troponin C. Most of the major peptides were eluted from the tryptic peptide map of the activator protein and subjected to amino acid analysis; of the peptides examined, only one peptide was found to have the identical amino acid composition as the corresponding peptide from the troponin C tryptic digest. This apparent lack of similarity between the peptide maps of protein activator and troponin C is in contrast to the striking similarities in amino acid composition and physical properties described in this paper. The data of Watterson et al.

on the tryptic peptides of the brain protein activator described in the accompanying paper (39) clearly show homology with troponin C. It should be emphasized here that closely related homologous proteins do not necessarily give very similar tryptic peptide maps. For example the protease inhibitors from lima bean and soybean have been shown by sequence studies to be about 70% homologous, yet they only have one tryptic peptide in common out of a possible total of six tryptic peptides (40). Similarly, sequence studies (37) have shown that there is 65% homology between rabbit skeletal and bovine cardiac troponin C; yet, based on comparing the amino acid sequences (31, 37) one would predict that their tryptic peptide maps would be quite different with only 3 out of a possible 18 peptides identical.

It has recently been demonstrated by Tuft and Kretsinger (41), by Collins (42), and also by Weeds and McLachlan (43) that there is structural homology between troponin C, the parvalbumins (a group of low molecular weight calcium-binding proteins), and myosin light chains. Evidence presented in this paper and the accompanying paper by Watterson et al. (39) allow us to add the bovine protein activator of phosphodiesterase to this family of calcium-binding proteins of apparently common evolutionary origin. Amongst these proteins only troponin C and the phosphodiesterase activator play a demonstrated role in calcium-mediated regulation of biological processes.

The sequence homology between bovine cardiac troponin C and rabbit skeletal muscle troponin C is only 65% (37) and this indicates that these proteins are more distantly related than the difference in species would suggest and van Eerd and Takahashi (37) have suggested that this sequence difference reflects a difference in tissue rather than a difference in species. This is in contrast to our own results on protein activator which indicate that the proteins from heart and brain are most likely identical in amino acid sequence.

Lehman et al. (16) have presented evidence for the existence of two types of regulation of muscle contraction, an actin-linked regulation involving the troponin system as typified by rabbit muscle and myosin-linked regulation as has been found in molluscan muscle; molluscan myosin is known to bind calcium and when combined with rabbit actin has a calcium-sensitive ATPase. The troponins are thus believed to have a restricted distribution in the animal kingdom; in our own studies we have found (17) that representative animal species of the major phyla all contain activating factors similar to the bovine activator of cyclic nucleotide phosphodiesterase described here. We are now in the process of isolating and purifying protein activator from lower species with the aim of determining their structural and evolutionary relationships. If these activators could be shown to be structurally related to the bovine activator it would be reasonable to assume that it could have been the precursor of troponin C. Furthermore, the wide occurrence of activator activity throughout evolution suggests that it serves a fundamental function; apart from its possible role in the regulation of cyclic nucleotide metabolism (1, 44) it has also been suggested as a working hypothesis that it may be involved in stimulus-secretion coupling (18).

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T. C. Vanaman, personal communication.
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bovine brain troponin C like calcium-binding protein and analyzed a sample of bovine heart activator.

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F C Stevens, M Walsh, H C Ho, T S Teo and J H Wang


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