AFFINITY CHROMATOGRAPHY PURIFICATION OF A CYCLIC NUCLEOTIDE PHOSPHODIESTERASE USING IMMOBILIZED MODULATOR PROTEIN, A TROPONIN C-LIKE PROTEIN FROM BRAIN

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SUMMARY: Modulator protein, a brain troponin C-like protein, has been coupled to Sepharose 4B using conditions that allow retention of phosphodiesterase stimulatory activity. This conjugate has been used to directly demonstrate the calcium dependent formation of a reversible modulator protein-phosphodiesterase complex and to purify a cyclic nucleotide phosphodiesterase by affinity chromatography.

INTRODUCTION: It has been reported that a ubiquitous calcium binding protein is present in relatively high levels in neurosecretory tissue of numerous vertebrate species (1,2,3) and is elevated in fibroblasts after transformation by RNA tumor viruses (4,5). This protein, referred to as modulator protein, is structurally similar to muscle troponin C (1,2,6), the calcium binding regulatory subunit of muscle actomyosin, and has at least three functions associated with it. In the presence of calcium, modulator protein will stimulate the activity of partially purified preparations of cyclic nucleotide phosphodiesterase (2,3,7,8), and the activity of purified preparations of Lubrol-solubilized adenyl cyclase (9,10,11). In addition, modulator protein has been shown to provide Ca^{2+} sensitivity to the ATPase activity of reconstituted skeletal muscle actomyosin [1], in a manner analogous to muscle troponin C.

Modulator protein appears to stimulate the activity of cyclic nucleotide phosphodiesterase preparations by the following proposed mechanism (6,7):

(1) Ca^{++} + Modulator $\rightleftharpoons$ (Ca^{++} - Modulator)

(2) (Ca^{++} - Modulator) + Enzyme $\rightleftharpoons$ (Enzyme - Ca^{++} - Modulator)

[1] Amphlett, G., Vanaman, T. C., and Perry, S. V. In preparation.

This report describes the preparation and use of a modulator protein - Sepharose 4B conjugate to directly demonstrate the reversible nature of this interaction. In addition, we have utilized this conjugate to purify an activatable form of cyclic nucleotide phosphodiesterase from bovine brain.

MATERIALS AND METHODS: Triethanolamine and ethanolamine were obtained from Eastman Organic Chemicals. Cyanogen bromide was obtained from Pierce Chemicals. Sepharose 4B was obtained from Pharmacia. All other chemicals were reagent grade and utilized without further purification.

Modulator protein was purified from bovine brain as previously described (2). Cyclic nucleotide phosphodiesterase activity and stimulation of "activator depleted" phosphodiesterase activity by modulator protein were assayed as previously described (2). Exact incubation conditions are given where appropriate in the text. Polyacrylamide gel electrophoresis was performed using previously described procedures (2,5).

A crude fraction of cyclic nucleotide phosphodiesterase activity that contained no detectable modulator protein was prepared from bovine brain. A homogenate supernatant fraction was prepared and chromatographed on DEAE-Sephadex A-25 exactly as previously described (2). All fractions eluting from the column prior to modulator protein, including the column wash with starting buffer, were pooled. Solid ammonium sulfate (313 grams/liter) was added to the pooled fractions to bring the solution to 50% saturation. The resulting solution was adjusted to pH 7.0 with 1 N NH_4OH, stirred for 1 hour at 4°, then centrifuged for 1 hour at 10,000 x g. The pellet was resuspended in 30-50 ml of deionized water and dissolved by adjusting the slurry to pH 7.0 with 1 M Tris base. The protein was extensively dialyzed at 4° against dialysis buffer (40 mM Tris-HCl, 1 mM 2-mercaptoethanol, 0.4 mM $MgCl_2$, pH 8.0). After dialysis the protein solution was stored frozen at -20° until ready for use.

Modulator protein was coupled to Sepharose 4B using essentially the procedure of March et al. (12). Briefly, 20 ml of Sepharose 4B slurry was washed with deionized water then treated with 1 gram of cyanogen bromide dissolved in 1 ml of acetonitrile. The slurry was stirred for 15 minutes at 0° and maintained at pH 11.0 by the dropwise addition of 2 M NaOH. After transferring to a sintered-glass funnel, the slurry was washed consecutively with 5-10 volumes each of deionized H_2O, 1 M triethanolamine-HCl, pH 8.5, and 0.05 M triethanolamine-HCl, pH 8.5, containing 1 mM $CaCl_2$ (buffer A). After the last wash, the slurry was filtered under suction to a moist cake, then transferred to an Erlenmeyer flask as a 1:1 slurry (total volume of 40 ml) using buffer A.

Coupling was performed at 4° for 8 hours. Modulator protein (5 mg) was dissolved in buffer A (5 ml) and added dropwise to the slurry with continuous stirring. After 8 hours the gel was washed with 40 ml of buffer A, then resuspended in 20 ml of 1 M ethanolamine-HCl, pH 8.0 and stirred for 2 hours at room temperature. The gel was then washed exhaustively with buffer B (40 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0), and stored as a 1:1 slurry in buffer B. A 500 µl aliquot of the slurry was hydrolyzed in 6 N HCl and the amount of modulator protein quantified by amino acid analysis as previously described (2).

RESULTS AND DISCUSSION: Based on amino acid analysis of acid hydrolyzed aliquots of the modulator protein-Sepharose 4B conjugate approximately one-half of the modulator protein added to the activated Sepharose slurry

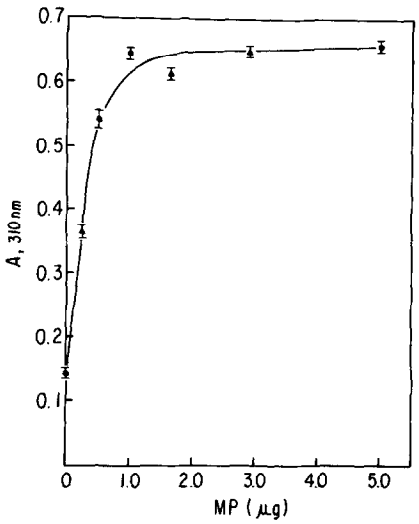

FIGURE 1. Activation of "activator-depleted" cyclic nucleotide phosphodiesterase from bovine brain by purified bovine brain modulator protein (●) and by the modulator protein-Sepharose 4B conjugate (▲). Assays were performed as described in the text. Reaction mixtures of 0.5 ml contained 40 mM Tris-HCl (pH 8.0), 0.4 mM $MnCl_2$, 1.0 mM $CaCl_2$, 2 mM cyclic AMP, 50 μg of bovine brain phosphodiesterase and the indicated amount of modulator protein (MP) quantified by amino acid analysis. Figures plotted are the mean values for phosphate determined in triplicate analyses. Error bars indicate the standard deviation from the mean.

was coupled to the resin. As shown in Figure 1, the modulator protein-Sepharose 4B conjugate is as active as the unmodified protein in stimulating "activator-depleted" bovine brain phosphodiesterase activity. Sepharose 4B alone and modulator protein cleaved with cyanogen bromide had no effect on phosphodiesterase activity in this system. The amino acid composition of the coupled protein was very similar to the unmodified material except that the lysine content was low as expected.

Chromatography of "activator-depleted" bovine brain phosphodiesterase on a column of the modulator protein-Sepharose 4B conjugate is shown in Figure 2. The sample was applied to the column in buffer containing 1 mM $CaCl_2$ and washed with this buffer until the absorbance at 280 nm (solid line) had returned to the baseline. Elution with an EGTA containing buffer was then commenced as indicated in the figure. Aliquots (100 μl) of even

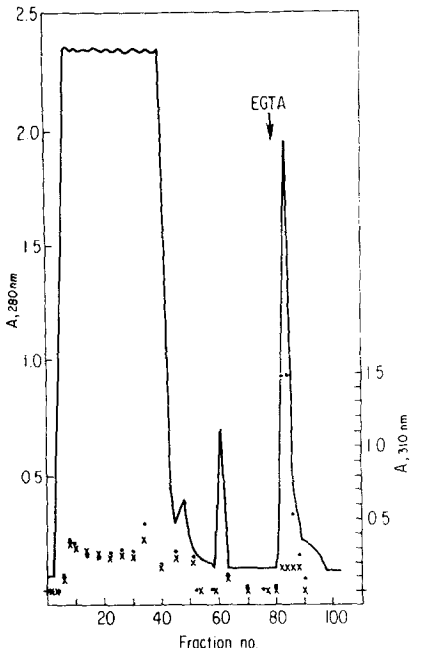

FIGURE 2. Chromatography using the modulator protein-Sepharose 4B conjugate. A 1 x 30 cm column was packed with modulator protein-Sepharose 4B conjugate and washed extensively with buffer B. The crude bovine brain phosphodiesterase preparation was applied to the column. Collection of fractions (2 ml each) was begun during sample loading. After the sample was applied to the column bed, the column was washed with buffer B containing 1 mM $CaCl_2$ until absorbance of the effluent at 280 nm returned to baseline. The elution buffer was changed to buffer B containing 1 mM EGTA (indicated by arrow) and absorbance of effluent fractions at 280 nm was monitored until the column had been eluted with four bed volumes of buffer. Even numbered fractions were assayed for phosphodiesterase activity (A, 310 nm) in the presence of excess calcium either with (•) or without (x) bovine brain modulator added to the assay mixture. Assays were performed as described in the legend to Figure 1.

numbered fractions were saved and frozen at -20° until assayed for phosphodiesterase activity. Clearly, a peak of 280 nm absorbing material was step eluted from the column by EGTA coincident with all the activatable phosphodiesterase activity. This material was separated from the bulk of the material applied to the column in a calcium containing buffer. After adding excess $CaCl_2$ to the peak fractions of the EGTA-eluted material, the activatable phosphodiesterase activity of these fractions was quantitatively absorbed to the same column and subsequently quantitatively eluted with

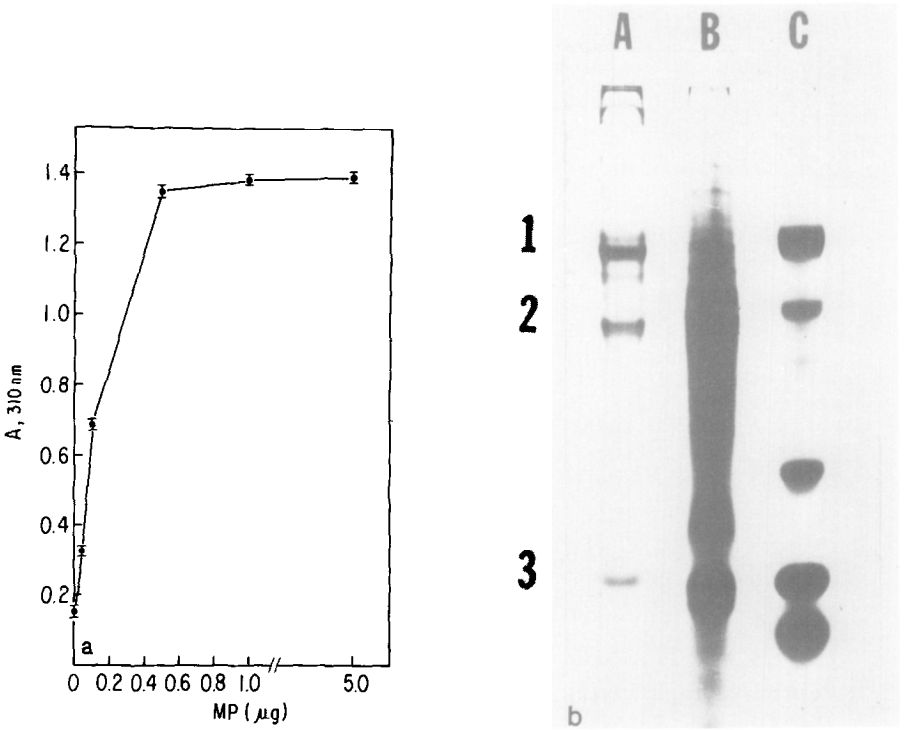

FIGURE 3. Analysis of purified phosphodiesterase. A. Activation of purified bovine brain phosphodiesterase by purified bovine brain modulator protein. Conditions of the assay are those described for Figure 1. Figures plotted are the mean values for phosphate determined in triplicate analyses. Error bars indicate the standard deviation from the mean. B. Sodium dodecylsulfate-polyacrylamide gel analysis. Samples, disrupted in 1% (w/v) sodium dodecylsulfate, 1% (v/v) 2-mercaptoethanol, 0.1M sodium phosphate, pH 7.2, were analyzed on a slab gel consisting of 10% (w/v) acrylamide, 0.22% (w/v) bisacrylamide and containing 0.1% (w/v) sodium dodecylsulfate-5M urea as described by Pett et al. (13). The gel was stained with Coomassie brilliant blue R250 and destained as described by Watterson et al. (2). Slot A, EGTA-eluted phosphodiesterase, 15 μg of protein. Slot B, crude phosphodiesterase before affinity chromatography, 100 μg of protein. Slot C, molecular weight standards including bovine serum albumin, ovalbumin, α-chymotrypsinogen, sperm whale myoglobin and cytochrome C.

EGTA buffer. No detectable material was absorbed to and eluted from a column of Sepharose 4B alone using identical procedures.

The ability of bovine brain phosphodiesterase, purified by this technique, to be stimulated by increasing amounts of purified bovine brain modulator protein is shown in Figure 3A. As can be readily seen, the eluted phosphodiesterase is stimulated in a manner similar to that previously re-

ported for crude "activator-depleted" phosphodiesterase from bovine brain (2). Cyclic GMP was also hydrolyzed by the purified phosphodiesterase and this hydrolysis was stimulated by the addition of bovine brain modulator protein in the presence of calcium.

Analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis of the phosphodiesterase preparation before and after purification with modulator protein-Sepharose 4B is shown in Figure 3B. The "affinity-purified" phosphodiesterase (slot A) is heterogeneous giving rise to three major bands (approximate chain weights of 60,000, 40,000, and 18,000 gms/mol compared to molecular weight standards, slot C) as well as a number of minor bands. The major Coomassie blue staining band (band 1) is greatly enriched in this preparation when compared to a much larger amount of the material similarly analyzed before affinity chromatography (slot B). Band 3 (18,000 molecular weight), seen in the "affinity-purified" phosphodiesterase, might represent bound modulator protein. However, no modulator protein was detected when this purified phosphodiesterase was analyzed by non-denaturing discontinuous gel electrophoresis as previously described (2,5).

The reversible formation of a calcium dependent complex between modulator protein and cyclic nucleotide phosphodiesterase has been directly demonstrated in this study. In addition, the feasibility of obtaining affinity based separations on modulator protein-Sepharose 4B conjugates has been demonstrated. If the modulator protein-Sepharose 4B conjugate has retained the ability to stimulate Lubrol-solubilized adenyl cyclase, it might also be used to purify activatable adenyl cyclase. Since modulator protein has been shown to function as a troponin C[1], affinity based purification of elements of the actin regulatory protein complex of non-muscle tissues also should be feasible using the modulator protein-Sepharose conjugate. These observations are an indication of the numerous potential uses of modulator protein-Sepharose conjugates in affinity-based purifications and for the direct demonstration of modulator protein-enzyme complexes.

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REFERENCES:

1. Vanaman, T.C., Harrelson, W.G., and Watterson, D.M. (1975) Fed. Proc., 34, 307.
2. Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F., and Vanaman, T.C. (1976) J. Biol. Chem., 251,4501-4513.
3. Cheung, W.Y., Lin, Y.M., Liu, Y.P., and Smoake, J.A. (1975) In: Cyclic Nucleotides in Disease (Weiss, B., ed.), p. 321-350, University Park Press, Baltimore.
4. Watterson, D.M. and Vanaman, T.C. (1976) Fed. Proc., 35, 1363.
5. Watterson, D.M., Van Eldik, L.J., Smith, R.E., and Vanaman, T.C. (1976) Proc. Natl. Acad. Sci., 73, 2711-2715.
6. Wang, J.H., Teo, T.S., Ho, H.C., and Stevens, F.C. (1975) Adv. Cyclic Nucleotide Res., 5, 179-194.
7. Teshima, Y. and Kakiuchi, S. (1974) Biochem. Biophys. Res. Commun., 56, 489-495.
8. Teo, T.S. and Wang, J.H. (1973) J. Biol. Chem., 248, 5950-5955.
9. Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lin, Y.M., and Tallant, E. (1975) Biochem. Biophy. Res. Commun., 66, 1055-1062.
10. Lynch, T.J., Tallant, E.A., and Cheung, W.Y. (1976) Biochem. Biophy. Res. Commun., 68, 616-625.
11. Brostrom, C.O., Hwang, Y.C., Brenkenridge, B.M., and Wolff, D.J. (1975) Proc. Natl. Acad. Sci., 72, 64-68.
12. March, S.C., Parikh, I., and Cuatrecasas, P. (1974) Anal. Biochem., 60, 149-152.
13. Pett, D.M., Vanaman, T.C., and Joklik, W.K. (1973) Virology, 52, 174-186.