Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol.* (in press).
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183.
Lee, J. C., Tweedy, N., & Timasheff, S. N. (1978) *Biochemistry, 17*, 2783.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Murphy, D. B., & Borisy, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2696.
Murphy, D. B., Johnson, K. A., & Borisy, G. G. (1977a) *J. Mol. Biol. 117*, 33.
Murphy, D. B., Vallee, R. B., & Borisy, G. G. (1977b) *Biochemistry 16*, 2598.
Oosawa, F., & Higashi, S. (1967) *Prog. Theor. Biol. 1*, 79.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10.
Penningroth, S. M., Cleveland, D. W., & Kirschner, M. W. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book C, pp 1233–1257, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Rosenbaum, J. L., Binder, L. I., Granett, S., Dentler, W. L., Snell, W., Sloboda, R. D., & Haimo, L. (1975) *Ann. N.Y. Acad. Sci. 253*, 147.
Ross, H., Olmsted, J. B., & Rosenbaum, J. L. (1975) *Tissue Cell 7*, 107.
Schacterle, G. R., & Pollack, R. L. (1973) *Anal. Biochem. 51*, 654.
Sherline, P., & Schiavone, K. (1977) *Science 198*, 1038.
Sherline, P., & Schiavone, K. (1978), *J. Cell Biol. 79*, R9.
Sloboda, R. D., Dentler, W. L., Bloodgood, R. A., Telzer, B. R., Granett, S., & Rosenbaum, J. L. (1976a) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book C, pp 1171–1212, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sloboda, R. D., Dentler, W. L., & Rosenbaum, J. L. (1976b) *Biochemistry 15*, 4497.
Sloboda, R. D., & Rosenbaum, J. L. (1977) *J. Cell Biol. 75* (part 2), 286a.
Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177.
Weisenberg, R. C. (1972) *Science 177*, 1104.
Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858.
Witman, G. B., Cleveland, D. W., Weingarten, M. D., & Kirschner, M. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4070.
Yamada, K. M., Spooner, B. S., & Wessells, N. K. (1971) *J. Cell Biol. 49*, 614.

Isolation and Characterization of Ca^{2+}-Dependent Modulator Protein from the Marine Invertebrate *Renilla reniformis*†

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ABSTRACT: An acidic, low molecular weight (18 400–19 100) protein capable of activating porcine brain phosphodiesterase in the presence of calcium has been purified 2700-fold from the anthozoan coelenterate, *Renilla reniformis*. The protein has physical, spectral, and chemical properties similar to those of modulator proteins isolated from mammalian species. Amino acid composition studies reveal no significant differences between the *Renilla* and mammalian modulator proteins. For example, we observed 1 mol of ϵ-*N*-trimethyllysine per mol of protein, no tryptophan or cysteine, and high levels of glutamic and aspartic acid residues. The protein from *Renilla* complexes with troponin I and T subunits in the presence of calcium and quantitatively replaces porcine brain modulator in the calcium-dependent activation of porcine brain phosphodiesterase. The protein has a high affinity for calcium as judged by the low levels of free calcium required for modulator-dependent activation of phosphodiesterase. The similarities in physical and chemical properties, high affinity for calcium, and identical calcium-dependent activities of this protein from *Renilla* (as compared with modulator protein purified from mammalian systems) suggest that a high degree of structural conservation has been retained in modulator proteins isolated from these diverse evolutionary forms.

During attempts to purify cyclic nucleotide phosphodiesterase from mammalian brain extracts, Cheung (1970, 1971) and Kakiuchi et al. (1970) independently discovered a protein activator for this enzyme, which, in addition to Ca^{2+}, was required for maximal activity. We will use the term modulator protein for this activator because of its multiple Ca^{2+}-dependent functions, as originally suggested by Watterson et al. (1976). Modulator protein was initially isolated to homogeneity from extracts of bovine brain and heart and characterized as an acidic and heat-stable Ca^{2+}-binding protein having a molecular weight in the range of 15 000–20 000 (Teo et al., 1973; Teo & Wang, 1973; Lin et al., 1974; Wolff & Brostrom, 1974).

Additional regulatory functions of modulator protein have recently been described including the activation of soluble preparations of brain adenylate cyclase in the presence of Ca^{2+} (Brostrom et al., 1975; Cheung et al., 1975) and the activation of a Ca^{2+}-dependent protein kinase found in smooth and skeletal muscle which results in the phosphorylation of myosin light chain (Yagi et al., 1978; Dabrowska et al., 1978;

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Waisman et al., 1978a). Phosphorylation of synaptosomal membrane fractions from rat cerebral cortex (Schulman & Greengard, 1978) and activation of erythrocyte membrane (Mg^{2+} + Ca^{2+})-dependent ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977) have also been found to involve modulator protein. In addition, there is recent evidence which suggests that modulator protein may play a role in chromosome movement during mitosis (Welsh et al., 1978). Since modulator protein appears to be an important physiological sensor for rapid Ca^{2+} transients, it would not be surprising if this protein were found to be involved in numerous other cellular events that depend on such transients.

In addition to the regulatory functions described above, modulator protein, isolated from several mammalian sources, has been shown to be structurally similar to troponin C of muscle suggesting that these two proteins may have evolved from a common ancestor (Watterson et al., 1976; Stevens et al., 1976; Dedman et al., 1978; Vanaman et al., 1977). The structural similarities of these two proteins has resulted in the observation that modulator protein will substitute for troponin C in restoring Ca^{2+} sensitivity to desensitized skeletal muscle actomyosin. Thus modulator protein will react with troponin subunits Tn I and Tn T to form a hybrid complex which can regulate muscle actomyosin ATPase activity in response to Ca^{2+} (Amphlett et al., 1976; Dedman et al., 1977a).

Modulator protein has been found to be widely distributed in the animal kingdom. For example, it has been purified from extracts of a number of mammalian tissues (Lin et al., 1974; Teo et al., 1973; Wolff & Siegel, 1972; Yagi et al., 1978; Dedman et al., 1977b), from chicken gizzard (Dabrowska et al., 1978), from electroplax of the electric eel (Childers & Siegel, 1975), and from an annelid (Waisman et al., 1978b). A comparison of the physicochemical properties of modulator protein from these various sources, along with recent structural information (Watterson et al., 1976; Dedman et al., 1978), suggests that the structural features of this protein have been highly conserved during evolution. In addition, Ca^{2+}-dependent modulator protein activity has been detected in dialyzed and heated tissue extracts of a number of invertebrates (Waisman et al., 1975, 1978b) and has also been found to occur in high concentrations in spermatozoa of various species ranging from invertebrates to human (Jones et al., 1978), suggesting that modulator protein is ubiquitous in the animal kingdom.

We report here the isolation and characterization of modulator protein from a marine anthozoan coelenterate, *Renilla reinformis*, and a comparison of its properties with previously isolated modulator proteins of higher organisms.

Materials and Methods

Purification. Six kilograms of *Renilla*, processed and frozen at −80 °C (Matthews et al., 1977) were extracted into 48 L of extraction buffer (1.5 mM Tris, 1 mM EDTA, 0.6 mM NaN_3, pH 7.8) by homogenization with a Tekmar homogenizer. The material was then centrifuged for 7 min at 7000 rpm in the GS-3 rotor of a Sorvall RC-2B centrifuge at 4 °C. The supernatant was then applied to 4 L of DEAE-cellulose equilibrated with the extraction buffer.

The DEAE was successively washed with 40 L of the extraction buffer plus 0.035 M NaCl, with 24 L of 0.015 M Tris, 0.01 M EDTA, and 0.6 mM NaN_3, pH 7.5, and with 20 L of 7.5 mM Tris, 5 mM EDTA, 0.6 mM NaN_3, and 0.1 M NaCl, pH 7.8. The 24-L wash was used in order to obtain proteins used in our bioluminescence studies. The modulator protein was then eluted with 7.5 mM Tris, 5 mM EDTA, 0.6 mM NaN_3, and 0.75 M NaCl, pH 7.0. Fractions of 400 mL were collected and assayed for activity, and active fractions were pooled.

The pooled material was heated to 90 °C for 5 min, cooled in an ice bath, and then centrifuged as above for 15 min. The pellet was discarded; solid $(NH_4)_2SO_4$ was added to the supernatant to achieve 50% saturation and then stirred for 1 h. This solution was then centrifuged for 15 min as described above. The pellet was discarded and $(NH_4)_2SO_4$ was added to the resultant supernatant to achieve 70% saturation. The solution was stirred for 1 h and then centrifuged for 15 min. $(NH_4)_2SO_4$ was added to the resultant supernatant to achieve 95–100% saturation. The solution was stirred for 1 h and centrifuged for 15 min. The final pellet contained >90% of the modulator protein activity. The pellet was resuspended in 7.5 mM Tris, 5 mM EDTA, and 0.6 mM NaN_3, pH 7.8, and dialyzed extensively against the same buffer.

The dialyzed sample was applied to a 2 × 20 cm DEAE-cellulose column equilibrated with the dialysis buffer and eluted with a 4-L gradient of 0–0.4 M NaCl in 7.5 mM Tris, 5 mM EDTA, and 0.6 mM NaN_3, pH 7.8.

Fractions from this second DEAE-cellulose column were assayed for activity and the active fractions were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, and dialyzed against 7.5 mM Tris, 5 mM EDTA, and 0.6 mM NaN_3, pH 7.8. The dialyzed sample was applied to a 2.9 × 145 cm Sephadex G-75 superfine column equilibrated in the same buffer. Three-milliliter fractions were collected and assayed for activity.

Assay of Modulator Protein Activity. Modulator protein activity was measured by the method of Matthews & Cormier (1978) and is based on the cyclic nucleotide phosphodiesterase assay of Weiss et al. (1972). The assay involves coupling the calcium and modulator protein-dependent activation of phosphodiesterase activity to the firefly luciferin–luciferase bioluminescence reaction. A portable photometer, designed and constructed by the Bioluminescence Laboratory, was used for routine luminescence assays (Anderson et al., 1978). One unit of modulator protein is defined as that amount of protein required to produce 50% maximal activation of that amount of phosphodiesterase which is sufficient to produce 1 nmol of AMP per min when saturated with modulator protein and Ca^{2+} at pH 8.0 at 25 °C.

Protein Determination. Protein concentration was determined by the method of Goa (1958) using bovine serum albumin as the standard.

Acrylamide Gel Electrophoresis. Discontinuous gel electrophoresis was carried out according to the method of Gabriel (1971) on 15% polyacrylamide gels. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted by the method of Weber & Osborn (1969). Binding studies on modulator protein and troponin components were conducted as described by Amphlett et al. (1976). Gels were stained with 0.04% (w/v) Coomassie Brilliant Blue G-250 in 3.5% (w/v) perchloric acid according to the method of Reisner et al. (1975). Gels were destained in 7.5% acetic acid. For protein quantitation, gels were stained with Amido black and scanned on a Cary 15 spectrometer.

Spectral Measurements. Spectra were recorded on an on-line spectrophotometer system constructed in the Bioluminescence Laboratory. The data represent the average of five scans as computed by a Data General Corp. Nova 820. Absorbance measurements were obtained using a Zeiss PMQ2 spectrometer.

Analytical Ultracentrifugation. Analytical ultracentrifugation was conducted with a Beckman-Spinco Model E analytical ultracentrifuge. Sedimentation equilibrium runs

Table I: Summary of Modulator Protein Purification

step	vol (mL)	protein (mg)	act. (total units)	sp act.	purification (X)	yield (%)
extraction	48 000	227 040	184 600	0.81		100
first DEAE	3 150	23 121	116 310	5.03	6.2	63
heat step + $(NH_4)_2SO_4$	72	1 058	92 250	87.1	107	50
second DEAE + $(NH_4)_2SO_4$	14.5	78.0	76 000	974.4	1200	41.2
G-75	96	35.5	78 000	2197	2700	42.3

were carried out at 20 °C at rotor speeds of 20 000 and 30 000 rpm. UV optics were used. The partial specific volume, $\bar{v}$, of the protein sample was calculated from the amino acid composition according to the method of Cohn & Edsall (1943). Sedimentation velocity experiments were conducted using Schlieren optics at 20 °C at rotor speeds of 68 000 and 60 000 rpm.

Amino Acid and Carbohydrate Analyses. Amino acid analyses were carried out on a Beckman Model 119 CL amino acid analyzer. Hydrolysates were prepared by heating the modulator protein under vacuum in 6 N HCl at 100 °C. Most amino acid determinations were made from 24-, 48- and 72-h hydrolysates of modulator protein by the method of Moore & Stein (1963). For composition analysis, time courses of hydrolysis were analyzed to correct for destructive losses of serine and threonine and for the slow release of valine and isoleucine. Tryptophan was determined by hydrolysis in the presence of 5% thioglycolate according to the method of Matsubara & Sasaki (1969). Cysteine was determined as cysteic acid from 24-h hydrolysates of performic acid oxidized modulator protein according to the method of Moore (1963). ϵ-*N*-Trimethyllysine was determined independently, following a 24-h hydrolysis, on a Beckman Model 119C amino acid analyzer using a modification of the standard Beckman program. The pH of the final buffer was changed to 5.4 and the program time extended by 20 min. Carbohydrate content of the modulator protein was determined, using glucose and ovalbumin as standards, with the phenol–sulfuric acid reaction (Dubois et al., 1956).

Ca^{2+} Titration Studies. Calcium titration studies were carried out by monitoring increases in phosphodiesterase activity as a function of free calcium concentration. Titrations were conducted in a Ca–EGTA buffer system containing 40 mM Tris and 2 mM magnesium acetate, pH 7.6. The modulator protein concentration in these experiments was 5.0×10^{-11} M.

Free calcium concentration in the Ca–EGTA buffer system was determined using a modified version of the program of Perrin & Sayce (1967), which was translated into Basic language. Association constants for the metals and H^+ to EGTA were obtained from Sillen & Martel (1964).

All chemicals utilized were of the best commercial grade available. Porcine modulator protein and phosphodiesterase were gifts of Dr. F. Siegel. Brain modulator protein and rabbit skeletal muscle troponin were gifts from Dr. T. C. Vanaman. The ϵ-*N*-trimethyllysine standard was a gift from Dr. R. Adelstein.

Results

Purification of Renilla Modulator Proteins. The calcium-dependent modulator protein of *Renilla reniformis* has been purified to homogeneity by the purification scheme described under Materials and Methods. As indicated in Table I, this procedure resulted in a 2700-fold purification of the modulator protein with a 42% recovery of activity. The purified protein has a specific activity of 2200 units/mg of protein. Based on the specific activity of the purified protein, the crude homogenate contained approximately 15 mg of modulator protein per kg of frozen *Renilla reniformis*.

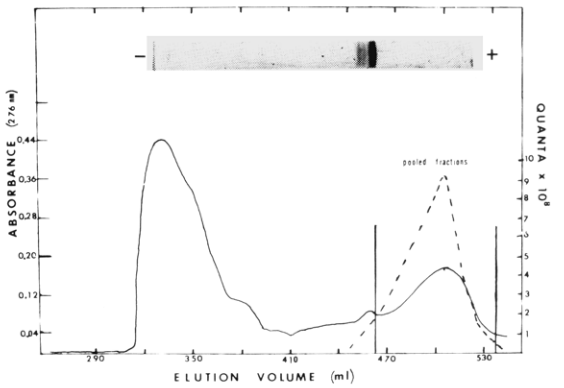

FIGURE 1: Column profile from Sephadex G-75 column. Protein was applied to a 2.9 × 145 cm Sephadex G-75 superfine column equilibrated in 7.5 mM Tris, 0.5 mM EDTA, and 0.6 M NaN_3, pH 7.8. Three-milliliter fractions were collected and assayed for protein as OD_{276} (—) and activity (- - -) was measured as the increase in emitted light in the bioluminescence assay system due to modulator protein. Active fractions were pooled as indicated. The inset shows the electrophoretic pattern of modulator protein from *Renilla reniformis*. A 75-μg sample of purified modulator protein from *Renilla reniformis* was electrophoresed on preelectrophoresed 15% polyacrylamide gels. Gels were stained with amido black and destained in 7% acetic acid.

Fractions from the second DEAE-cellulose column, which were found to contain modulator protein activity, were pooled. As was expected, the modulator protein eluted late in the gradient (0.3 M NaCl), consistent with the properties of a highly acidic protein.

Gel filtration of the modulator protein on a Sephadex G-75 superfine column resulted in the elution profile shown in Figure 1. Activity was observed coincident with a protein peak which eluted between 460 and 550 mL. The material was pooled and analyzed for purity and activity.

Purity of the Modulator Protein. The purity of the modulator protein was measured by discontinuous gel electrophoresis on 15% polyacrylamide gels under nondenaturing conditions. As shown in Figure 1 (inset), three protein bands were observed. Densiometric tracing of the gels indicated that approximately 90% of the protein was present in the major band. Slicing of the polyacrylamide gels and subsequent extraction and assay of the protein material present in each of the bands indicated that all three bands contained modulator protein activity. Purified bovine brain modulator protein also exhibits multiple bands under these electrophoretic conditions (Watterson et al., 1976). Thus, both the *Renilla* and bovine brain modulator proteins behave similarly during gel electrophoresis. Watterson et al. (1976) suggested that the multiple bands are due to aggregate formation. We found that a single modulator protein band was observed upon gel electrophoresis in the presence of calcium. This band corresponded in position to the slowest moving, minor modulator protein band seen in the inset (Figure 1). A single protein band

Table II: Physical and Spectral Comparisons of Modulator Protein from *Renilla* and Bovine Heart and Brain

parameter	*Renilla*	bovine heart	bovine brain
sedimentation coeff(s)	1.5	2.0[a]	
mol wt			
anal. ultracentrifugation	18 400–19 100	17 000–19 000[a]	17 700–17 850[c]
$NaDodSO_4$ gel electrophoresis	15 600	18 000[a]	18 000[c]
gel filtration	25 000	27 000[b]	
$E_{275-278}$ (1% protein, 1 cm)	1.9	1.9[a]	1.8[c]
absorption max (nm)	252, 258, 264, 268, 276	252, 258, 264, 267, 275[a]	252, 258, 264, 267, 276[c]

[a] From Stevens et al. (1976). [b] From Teo et al. (1973). [c] From Watterson et al. (1976).

was observed upon electrophoresis in the presence of $NaDodSO_4$. These data indicate that the modulator protein is essentially pure.

Physical and Spectral Properties. The molecular weight of the modulator protein was determined by sedimentation equilibrium studies in 7.5 mM Tris, 5 mM EDTA, and 200 mM NaCl, pH 7.0, at 20 °C. Linear plots of ln c vs. r^2 with correlation coefficients of >0.99 were obtained at both 20 000 and 30 000 rpm. The slopes of these lines were 0.496 and 1.042, respectively, resulting in calculated molecular weights of 19 143 ± 345 and 18 365 ± 410.

$NaDodSO_4$ gel electrophoresis of the modulator protein on 10% polyacrylamide gels using ovalbumin, whale skeletal muscle myoglobin, soybean trypsin inhibitor, and cytochrome c as standards showed the modulator protein to be composed of a single polypeptide chain of molecular weight 15 600 ± 200.

The Stokes' radius of the modulator protein was determined by gel filtration on Sephadex G-75 superfine in 10 mM Tris, 0.1 mM EDTA, and 0.2 M KCl, pH 8.0, utilizing ovalbumin, soybean trypsin inhibitor, horse heart cytochrome c, and whale skeletal muscle myoglobin as standards and blue dextran and thiamine as markers of excluded and included volume, respectively. Samples of the standards and the modulator protein were chromatographed in triplicate. The data obtained by this method were plotted as $K_D^{1/3}$ vs. Stokes' radius resulting in a linear plot with a correlation coefficient of 0.98 (Porath, 1963). The Stokes' radius for the modulator protein as determined by this method was 22.4 Å in both the presence and absence of 10 mM $CaCl_2$. In contrast, Kuo & Coffee (1976) noted slight differences in the Ca^{2+}-bound and Ca^{2+}-free forms of modulator protein isolated from bovine adrenal medulla. The observed Stokes' radius for the *Renilla* modulator protein was larger than that expected for a globular protein of molecular weight 18 500 and suggests some degree of asymmetry within the molecule.

The sedimentation coefficient of the *Renilla* modulator protein was determined by ultracentrifugation at concentrations of 10, 7.5, and 5.6 mg/mL at 68 000 rpm in 10 mM Tris, 1 mM EDTA, and 0.2 M NaCl, pH 7.8. The results of these studies revealed a sedimentation coefficient ($s_{20,w}^0$) for the modulator protein of 1.5 S.

The ultraviolet absorption spectrum of the *Renilla* modulator protein was found to be characteristic of modulator protein purified from other species with absorption maxima observed at 276, 268, 264, 258, and 252 nm. The molar extinction coefficient determined for the modulator protein in 7.5 mM Tris, 5 mM EDTA, and 0.6 mM NaN_3, pH 7.8, at 276 nm was 3384 which is equivalent to an $\epsilon_{276}^{1\%}$ of 1.9.

A comparison of the physical and spectral features of the modulator protein obtained from *Renilla* to the modulator protein obtained from bovine brain is presented in Table II. In each of the studies conducted, the *Renilla* modulator protein was found to be strikingly similar, if not identical, to the modulator protein isolated from bovine brain.

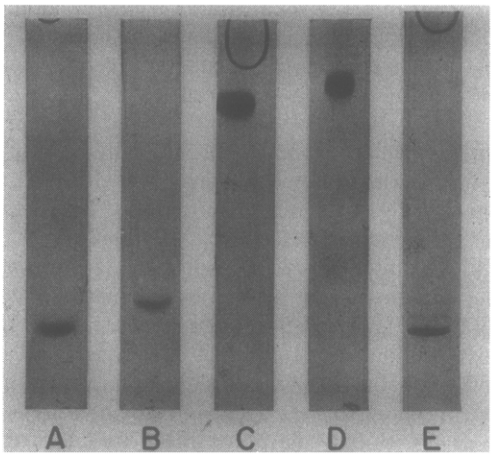

FIGURE 2: Interaction of modulator protein from *Renilla reniformis* and troponin C with troponin I. Troponin I–modulator protein and troponin I–troponin C interactions were determined by gel electrophoresis by the method of Amphlett et al. (1976). (A) Troponin C + calcium. (B) *Renilla* modulator + calcium. (C) Troponin I + troponin C + calcium. (D) Troponin I + *Renilla* modulator protein + calcium. (E) Troponin I + *Renilla* modulator protein + EDTA.

Activity Comparisons. The ability of the *Renilla* and bovine brain modulator proteins to stimulate brain phosphodiesterase activity in the presence of calcium was compared in a series of experiments. The saturation curves obtained for the *Renilla* modulator protein and the modulator protein from bovine brain were essentially the same with a maximal 3.7-fold stimulation of phosphodiesterase activity and a half-saturation concentration of 0.05 μg of modulator protein observed for both modulators under these experimental conditions. EGTA was found to totally inhibit this activation which is consistent with the calcium requirement of the modulator protein.

Amphlett et al. (1976) have demonstrated that bovine brain modulator protein (in the presence of calcium) can successfully replace troponin C in the formation of protein complexes with troponin subunits Tn I and Tn T. The same phenomenon has been observed with rat testes modulator protein (Dedman et al., 1977a). The ability of the *Renilla* modulator protein to bind to these troponin subunits was also investigated. As is shown in Figure 2, *Renilla* modulator protein complexed with Tn I in the presence of calcium in a manner similar to that observed for troponin C. In the presence of EGTA no complex was formed, indicating a calcium requirement for complex formation. Similar calcium-dependent complex formation was observed with mixtures of Tn T and modulator protein. Therefore, as is the case with the bovine brain and rat testes modulator proteins, the *Renilla* modulator protein can form complexes with Tn I and Tn T in the presence of calcium.

Amino Acid and Carbohydrate Analyses. The results of the amino acid analysis shown in Table III were in excellent agreement with the physical characteristics described for the

Table III: Comparative Amino Acid Compositions of Modulator Proteins

	Renilla modulator[a] (mol/18750g)	nearest integer	bovine brain modulator[b]	rat testis modulator[c]	*Lumbricus terrestris* modulator[d]
Lys	9.21	9	8	7	7
His	1.50	1–2	1	1	1–2
Me_3-Lys	1.02	1	1[e]	1	*f*
Arg	5.84	6	7	6	5
Asp	26.23	26	24	22	24
Thr	12.51	13	12	11	12
Ser	6.25	6	5	4	6
Glu	30.90	31	29	28	29
Pro	1.60	2	2	2	6
Gly	12.55	13	12	12	13
Ala	10.93	11	12	11	11
$^1/_2$-cystine	0.0	0	0	0	1
Val	7.32	7	8	8	7
Met	8.70	9	10	9	9
Ile	8.75	9	8	9	8
Leu	10.74	11	10	10	10
Tyr	1.60	2	2	2	2
Phe	9.12	9	8	8	8
Trp	0.0	0	0	0	0

[a] All values were calculated as the average of determinations made following 24-, 48-, and 72-h hydrolyses except as noted in Materials and Methods. [b] From Watterson et al., 1976. [c] From Dedman et al., 1977b. [d] From Waisman et al., 1978b. [e] Originally reported as compound X by Watterson et al. (1976) and later identified as trimethyllysine by Jackson et al. (1977). [f] Trimethyllysine was not resolved from lysine in the reported amino acid analysis of Waisman et al. (1978b).

Renilla modulator protein. The high number of aspartic and glutamic acid residues is consistent with the large net negative charge observed for the protein upon gel electrophoresis, and the absence of tryptophan and high ratio of phenylalanine to tyrosine were consistent with the observed spectrum.

The modulator protein from *Renilla* was found to be remarkably similar to modulator proteins isolated from other sources including the presence of 1 mol of the modified amino acid trimethyllysine per mol of protein, no tryptophan residues, and a relative preponderance of negatively charged amino acids. As is the case with the bovine brain and rat testes modulator proteins, no cysteine and two proline residues occurred in the protein. Within the limits of the analysis, there were no significant differences between the *Renilla* modulator protein and those isolated from bovine brain and rat testes. Consistent with the findings for other modulator proteins, no observable carbohydrate was present in the purified *Renilla* modulator protein.

Ca^{2+} Dependence of Activation. The modulator protein-specific activation of porcine brain phosphodiesterase by *Renilla* modulator protein was determined at various levels of free Ca^{2+} in order to establish the levels of Ca^{2+} required for activation. As shown in Figure 3, Ca^{2+}-dependent activation of phosphodiesterase, in the presence of the *Renilla* modulator protein, occurred at free Ca^{2+} levels in the range of 10^{-7} M. Under the same conditions bovine brain modulator protein was shown to be activated at these same levels of Ca^{2+}. The required levels of free Ca^{2+} for activation in this system were slightly lower than those reported for modulator proteins in other systems (Watterson et al., 1976; Dabrowska et al., 1978; Dedman et al., 1977b). The observed differences may be explainable, however, by differences in the ionic strengths of the buffers used in the various systems. However, these data

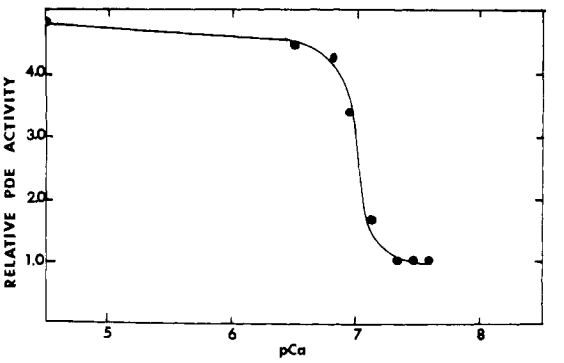

FIGURE 3: Calcium dependence of phosphodiesterase activation by *Renilla* modulator protein. Differences in phosphodiesterase activation by *Renilla* modulator protein in the presence of various levels of free calcium were measured. The amount of modulator protein used in these experiments was tenfold in excess of saturation. Details of the experiment are included in the text.

do conclusively show that the modulator specific activation of phosphodiesterase by *Renilla* modulator is triggered at extremely low levels of free Ca^{2+}. This is consistent with the observation of high affinity binding sites for Ca^{2+} observed in all modulator proteins reported to data.

Discussion

The anthozoan coelenterate, *Renilla reniformis*, represents the lowest evolutionary form of life which has been used as a source material for the isolation of modulator protein. An examination of the physical properties of this protein, its biological activity with cyclic nucleotide phosphodiesterase, and interaction with troponin subunits supports earlier suggestions that the modulator protein is ubiquitous in the animal kingdom[1] and that its structural features have been highly conserved during evolution. Absorption spectra, physical characteristics, and amino acid compositions of modulator proteins from *Renilla* and from mammalian species are (within experimental error) indistiguishable. Modulator protein from either source is characterized by the lack of cysteine and tryptophan and content of 1 mol of ϵ-*N*-trimethyllysine per mol of protein. These similarities suggest that it would be of interest to compare the amino acid sequence of the modulator protein from *Renilla* and the previously sequenced bovine brain and rat testes modulator proteins (Vanaman et al., 1977; Dedman et al., 1978). Studies of this nature are presently in progress.

The results of the Ca^{2+} activation studies suggest that the *Renilla* modulator protein, like mammalian modulator proteins, is well suited to play a regulatory role as a Ca^{2+} sensor for intracellular Ca^{2+} transients. As in other nonmammalian systems (Childers & Siegel, 1975; Waisman et al., 1978b), we have found no endogenous modulator protein-sensitive phosphodiesterase in extracts of *Renilla reniformis*, suggesting that modulator protein must serve other Ca^{2+}-dependent functions in this organism. Among the Ca^{2+}-regulated systems in *Renilla* is the process of bioluminescence. In that system a Ca^{2+}-binding protein which is similar, but not identical, to modulator protein serves as the terminal link between nerve excitation and the bioluminescence flash (Cormier & Charbonneau, 1977). It will be of interest to determine the

[1] Waisman et al. (1978b) have recently detected modulator protein activity in several species of higher plants. We too have noted the presence of a low molecular weight, heat-stable, Ca^{2+}-dependent activator of porcine brain cyclic nucleotide phosphodiesterase in several species of higher plants. (Anderson & Cormier, 1978).

possible role of modulator protein in the control of the bioluminescence flash in this animal especially in regard to its potential regulatory role in the resequestering of Ca^{2+} in this system.

References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72*, 163.

Anderson, J. M., & Cormier, M. J. (1978) *Biochem. Biophys. Res. Commun. 84*, 595.

Anderson, J. M., Faini, G. J., & Wampler, J. E. (1979) *Methods Enzymol.* (in press).

Brostrom, C. O., Huang, Y.-C., Breckenridge, B. M., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64.

Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun. 38*, 533.

Cheung, W. Y. (1971) *J. Biol. Chem. 246*, 2859.

Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun. 66*, 1055.

Childers, S. R., & Siegel, F. L. (1975) *Biochim. Biophys. Acta 405*, 99.

Cohn, E. J. & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides*, p 375, Reinhold, New York.

Cormier, M. J., & Charbonneau, H. C. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) p 481, North-Holland, New York.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Harshorne, D. J. (1978) *Biochemistry 17*, 253.

Dedman, J. R. Potter, J. P., & Means, A. R. (1977a) *J. Biol. Chem. 252*, 2437.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977b) *J. Biol. Chem. 252*, 8415.

Dedman, J. R., Jackson, R. L., Schreiber, W. E., & Means, A. R. (1978) *J. Biol. Chem. 253*, 343.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebens, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.

Gabriel, O. (1971) *Methods Enzymol. 22*, 565.

Goa, J. (1958) *Scand. J. Clin. Lab. Invest. 5*, 218.

Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77*, 1203.

Jackson, R. L., Dedman, J. R., Schreiber, W. E., Bhatnagar, R. K., Knapp, R. D., & Means, A. R. (1977) *Biochem. Biophys. Res. Commun. 77*, 723.

Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77*, 1210.

Jones, H. P., Bradford, M. M., McRorie, R. A., & Cormier, M. J. (1978) *Biochem. Biophys. Res. Commun. 82*, 1264.

Kakiuchi, S., Yamazaki, R., & Nakajima, H. (1970) *Proc. Jpn. Acad. 46*, 587.

Kuo, I. C. K., & Coffee, C. J. (1976) *J. Biol. Chem. 251*, 6315.

Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974) *J. Biol. Chem. 249*, 4943.

Matsubara, H., & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun. 35*, 175.

Matthews, J. C., & Cormier, M. J. (1978) *Methods Enzymol.* (in press).

Matthews, J. C., Hori, K., & Cormier, M. J. (1977) *Biochemistry 16*, 85.

Moore, S. (1963) *J. Biol. Chem. 238*, 235.

Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819.

Perrin, D. D., & Sayce, I. G. (1967) *Talanta 14*, 833.

Porath, J. (1963) *Pure Appl. Chem. 6*, 233.

Reisner, A. H., Nemes, P., & Bucholtz, C. (1975) *Anal. Biochem. 64*, 509.

Schulman, H., & Greengard, P. (1978) *Nature (London) 271*, 478.

Sillen, L. G., & Martell, A. E. (1964) *Chem. Soc., Spec. Publ. No. 17*, 1.

Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S., & Wang, J. H. (1976) *J. Biol. Chem. 251*, 4495.

Teo, T. S., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 5950.

Teo, T. S., Wang, T. H., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 588.

Vanaman, T. C., Sharief, F., & Watterson, D. M. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) p 107, North-Holland, New York.

Waisman, D., Stevens, F. C., & Wang, J. H. (1975) *Biochem. Biophys. Res. Commun. 65*, 975.

Waisman, D. M., Singh, T. J., & Wang, J. H. (1978a) *J. Biol. Chem. 253*, 3387.

Wasiman, D. M., Stevens, F. C., & Wang, J. H. (1978b) *J. Biol. Chem. 253*, 1106.

Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Weiss, B., Lehne, R., & Strada, S. (1972) *Anal. Biochem. 45*, 222.

Welsh, M. J., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1867.

Wolff, D. J., & Siegel, F. L. (1972) *J. Biol. Chem. 247*, 4180.

Wolff, D. J., & Brostrom, C. O. (1974) *Arch. Biochem. Biophys. 163*, 349.

Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., & Uenishi, K. (1978) *J. Biol. Chem. 253*, 1338.