Isolation of a 5.3-S calmodulin-deficient 3':5'-cyclic nucleotide phosphodiesterase from cardiac muscle

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1. In the presence of Ca^{2+} , a 5.3-S 3':5'-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) from bovine ventricle was isolated and purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose and Affi-Gel Blue chromatography. The enzyme activity was enriched 800-fold by these procedures.
2. Sucrose-density-gradient centrifugation, gel filtration and non-denaturing polyacrylamide-gel electrophoresis resolved a single enzyme species with an M_r of 89 000.
3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified enzyme demonstrated a prominent protein band at M_r 59 000 and a minor band of M_r 28 000. Calmodulin was not detected.
4. The hydrolysis of μmolar concentrations of 3':5'-cyclic guanosine monophosphate (cyclic GMP) but not 3':5'-cyclic adenosine monophosphate (cyclic AMP) was stimulated by calmodulin.
5. Anomalous biphasic kinetic plots were observed for both the catalysis of cyclic AMP and cyclic GMP hydrolysis. Kinetic plots became linear in the presence of calmodulin.
6. After several months of storage at -20°C , the 5.3-S enzyme was transformed into a 6.2-S cyclic GMP-specific enzyme and a 4.4-S non-specific form.

The multiple forms of cytosolic 3':5'-cyclic nucleotide monophosphate phosphodiesterase in mammalian tissues may result from association with calmodulin (Cheung, 1971; Kakiuchi *et al.*, 1971), endogenous proteolytic action (Epstein *et al.*, 1978; Tucker *et al.*, 1981) and perhaps from solubilization of membrane-bound forms (Beavo *et al.*, 1970). Enzymes with discrete specificities for cyclic AMP (Thompson *et al.*, 1979), cyclic GMP (Davis & Kuo, 1977) and cyclic CMP (Helfman *et al.*, 1981) have been reported, but structural properties remain to be determined. Phosphodiesterases responsive to calmodulin are more specific for cyclic GMP as substrate than cyclic AMP (Kakiuchi *et al.*, 1973), whereas calmodulin-insensitive enzymes are often specific for cyclic AMP (Keravis *et al.*, 1980). A characteristic structural feature of calmodulin-dependent phosphodiesterase is a catalytic subunit of mol.wt. 57 000–63 000 (Klee *et al.*, 1979; La Porte *et al.*, 1979).

Current isolation procedures for phosphodiesterases use Ca^{2+} -free, calmodulin-dissociating conditions to purify calmodulin-dependent phosphodiesterases. With cardiac tissue, a calmodulin-insensitive phosphodiesterase is often observed during isolation procedures employing EGTA (Ho *et al.*, 1977; Mohindru *et al.*, 1978). To further investigate the properties of enzymes prepared by different

procedures, we purified the cardiac phosphodiesterase in buffers supplemented with low Ca^{2+} and without exposure to EGTA. Herein, we report on the catalytic and physicochemical properties of a calmodulin-deficient 5.3-S enzyme isolated by this procedure.

Materials and methods

Materials

3':5'-Cyclic [8- ^3H]AMP (sp. radioactivity 21 Ci/mmol) and 3':5'-cyclic [8- ^3H]GMP (sp. radioactivity 8.8 Ci/mmol) were purchased from Schwarz Mann, Orangeburg, NY, U.S.A. The radioisotopes were purified on DEAE-Sephadex A-25 before use. Bovine serum albumin and other protein standards were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex resins and Blue Dextran 2000 were purchased from Pharmacia, Piscataway, NJ, U.S.A. Affi-Gel Blue agarose and Chelex-100 were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Methods

Phosphodiesterase assay. The radiometric assay of Thompson & Appleman (1971) was used to measure activity within a substrate concentration range of 0.1–100 μM . The assay mixtures contained

40 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂, 0.1 mg of bovine serum albumin/ml, (8-³H)-labelled cyclic nucleotide and enzyme in a total volume of 0.5 ml. The procedure for the isolation of product and other conditions were as previously described (Mohindru *et al.*, 1978). Enzyme activity at 1 mM substrate was determined colorimetrically by the release of P_i (Butcher & Sutherland, 1962). Units of activity are defined as μ mol of cyclic nucleotide hydrolysed per min at 30°C. Protein was determined by the Coomassie Blue G binding assay of Bradford (1976) with crystalline bovine serum albumin as standard.

Estimation of molecular weight. Molecular weight was estimated by the gel-electrophoresis procedure described by Hedrick & Smith (1968). The concentration of acrylamide was varied from 4 to 8% and the gels were supplemented with 0.5 mM-dithiothreitol and 5 mM-MgCl₂. After electrophoresis (5 mA/tube) at 4°C, the gels were sliced into 15 equal segments (2–3 mm). Each segment was extracted for 24 h at 4°C with 200 μ l of 50 mM-Tris/HCl, pH 7.4, containing 0.5 mM-dithiothreitol and 5 mM-MgCl₂ and assayed for cyclic AMP and cyclic GMP activity by radiometric assay.

Determination of the sedimentation coefficient and estimation of the molecular weight by sucrose-density-gradient centrifugation were performed by the procedure of Martin & Ames (1961). The gradients ranged from 18 to 38% (w/w) sucrose. A 0.2 ml sample was layered on to the gradient, which contained 50 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂ and 0.5 mM-dithiothreitol and was centrifuged at 50000 rev./min in a Beckman 50.1 rotor for 18 h at 5°C. Catalase (11.3 S), ovalbumin (3.6 S), bovine serum albumin (4.5 S) and lactate dehydrogenase (6.9 S) served as standards.

Molecular-weight estimates by chromatography on Sephadex G-100 were performed by the procedure of Andrews (1964) on a 2.6 cm $\times$ 52 cm column. The void volume was determined with Blue Dextran 2000.

Purification of cardiac phosphodiesterase. Fresh bovine cardiac ventricles were homogenized in 3 vol. of 0.25 M-sucrose containing 1 mM-MgCl₂ and 0.3 mM-phenylmethanesulphonyl fluoride in a Waring blender and centrifuged at 12000 g for 30 min. The supernatant was collected and adjusted to pH 7.4 and (NH₄)₂SO₄ was added to 55% saturation. After 30 min of centrifugation at 12000 g, the pellet was resuspended in 25 mM-Tris/HCl, pH 7.4, containing 1 mM-MgCl₂, 0.5 mM-dithiothreitol and 100 μ M-CaCl₂ (buffer A) and desalted on a column of Sephadex G-25 equilibrated with this same buffer. The enzyme was then applied to a column (2.6 cm $\times$ 30 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with 275 ml of buffer A containing 60 mM-NaCl and eluted with a 600-ml linear gradient from

60 to 500 mM-NaCl. The pooled fractions were applied to a column (1.6 cm $\times$ 10 cm) of Affi-Gel Blue agarose. Unadsorbed protein was washed from the column with buffer A followed by 2.0 M-NaCl in buffer A. Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities were then eluted as a single peak with 1 mM-cyclic AMP in buffer A containing 1.5 M-NaCl. These fractions were pooled and desalted on a Sephadex G-25 column (2.6 cm $\times$ 40 cm) equilibrated with buffer A. All of the above procedures were performed at 4°C. The purified enzyme was stored at -20°C in 10% (w/w) sucrose in buffer A.

The sensitivity of the enzyme to calmodulin (4 μ g/ml) or EGTA (250 μ M) was determined by comparison with the basal activity measured in the presence of Ca²⁺ (250 μ M) at 1 μ M substrate. For experiments involving EGTA/Ca²⁺ buffers, the enzyme was extensively dialysed against 250 μ M-EGTA followed by dialysis against 10 vol. of Chelex-treated deionized water. The EGTA/Ca²⁺ buffer was prepared utilizing the binding constants of Rodan & Feinstein (1976) as described by Bartfai (1978).

Purification and measurement of calmodulin. Calmodulin was purified from bovine cerebral cortex by the procedure of Watterson *et al.* (1976). The preparation was judged homogeneous by electrophoresis on discontinuous polyacrylamide gels at pH 8.9 (Davis, 1964) and at pH 4.3 (Reisfield *et al.*, 1962) as well as on sodium dodecyl sulphate-containing gels (Laemmli, 1970).

Calmodulin was assayed by the method of Cheung (1971) by measuring the stimulated and basal hydrolytic rates of calmodulin-deficient phosphodiesterase prepared from cerebral cortex. One unit of calmodulin activity was defined as the amount that caused a 50% Ca²⁺-dependent activation of the calmodulin-deficient enzyme.

Results

Purification of phosphodiesterase

Cardiac phosphodiesterase activity measured at 1 mM-cyclic AMP reproducibly eluted as a single symmetrical peak from the DEAE-cellulose and Affi-Gel Blue agarose (Fig. 1) equilibrated with Ca²⁺-supplemented buffer. Phosphodiesterase activity was eluted from Affi-Gel Blue agarose with 1 mM-cyclic AMP and 1.5 M-NaCl in buffer A as shown in Fig. 1. The enzyme could not be eluted by 2.0 M-NaCl or by buffer A containing either 1 mM-cyclic AMP, -5'-AMP or -EDTA. The overall purification of the enzyme was approx. 800-fold with a 9.0% yield in activity (Table 1). The extent of purification ranged from 300 to 1000-fold among preparations. Further purification by a variety of

techniques proved unsuccessful under our conditions.

The purified protein fraction contained two proteins by non-denaturing polyacrylamide-gel electrophoresis at pH 8.9 (Fig. 2). Phosphodiesterase activity was coincident with the light protein-staining band (R_F , 0.26) and was 12% pure by densitometric analysis. The predominant protein-staining band (R_F , 0.52) did not have phosphodiesterase activity. A protein-staining band corresponding to calmodulin was not observed on these gels. The gel region corresponding to phosphodiesterase activity was cut from 16 separate non-denaturing gels and extracted by incubation at 4°C for 24 h in buffer A. The extracted protein was electrophoresed on 10% sodium dodecyl sulphate/polyacrylamide gels (Laemmli, 1970). A prominent protein-staining band of M_r 59 000 was reproducibly observed (Fig. 3, gel *a*) for the phosphodiesterase activity region extracted from non-denaturing gels. An additional faint-staining protein in the region of M_r 28 000 was also consistently

present. This band produced only 25% of the staining intensity of the 59 000- M_r protein based on densitometric analysis. Some cross-contamination occurred in the region of M_r 32 000–38 000 from the predominant co-purifying protein. A protein-staining band corresponding to calmodulin was not observed. Calmodulin activity was also undetectable by direct

Fig. 1. Chromatography of cardiac phosphodiesterase on Affi-Gel Blue-agarose

The pooled enzyme activity (397 mg of protein) from the DEAE-cellulose column was applied to a column (1.6 cm × 10 cm) of Affi-Gel Blue. Phosphodiesterase activity (Δ) was measured by P_i release and protein (●) at 235 nm. Elution with 2.0 M-NaCl in buffer A (150 ml) was started at arrow A and elution with 1 μM-cyclic AMP and 1.5 M-NaCl in buffer A (100 ml) at arrow B.

Fig. 2. Non-denaturing polyacrylamide-gel electrophoresis of phosphodiesterase from Affi-Gel Blue agarose chromatography

Protein (28.8 μg) was analysed on 7% polyacrylamide gels (Davis, 1964). Coomassie Blue stain was quantified by densitometric tracing at 600 nm (●; *a*). Gels were also sliced, eluted and monitored for phosphodiesterase activity (*b*) at 0.1 μM-cyclic AMP (●) and 0.1 μM-cyclic GMP (○) utilizing the radiometric assay.

Table 1. Purification of 3':5'-cyclic nucleotide phosphodiesterase of bovine cardiac ventricle
Values are based on 1 kg of tissue and are averages of three separate preparations.

Step	Total protein (mg)	Total units* (m-units)	Specific activity (m-units/mg of protein)	Yield (%)	Purification (fold)
12 000 g supernatant	21 000	3180	0.150	100	1.00
(NH ₄) ₂ SO ₄	3030	2050	0.675	64.5	4.50
DEAE-cellulose	397	877	2.21	42.9	15.2
Affi-Gel Blue	2.4	285	118	8.9	790

* As measured by the hydrolysis of 1.0 μM-cyclic AMP by the radiometric assay. Results are corrected for adenosine recovery.

assay utilizing calmodulin-deficient phosphodiesterase purified from brain. The predominant co-purifying protein observed on non-denaturing gels (R_F 0.52) was also extracted and subjected to sodium dodecylsulphate/polyacrylamide-gel electrophoresis. As seen in Fig. 3 (gel *b*), three unidentified bands corresponding to molecular weights from 32 000 to 38 000 were associated with this protein.

Estimation of apparent molecular weight

Sucrose-density-gradient analysis of the enzyme from Affi-Gel Blue agarose yielded an average sedimentation coefficient of 5.3-S, which corresponded to an approximate molecular weight $\pm$ S.D. of $86\,000 \pm 5\,000$ (14 determinations). Both cyclic AMP and cyclic GMP hydrolytic activities migrated as a single symmetrical peak; the position of this peak was not influenced by the addition of $250\,\mu\text{M}$ -EGTA to the gradient.

The molecular weight of the 5.3-S enzyme from the Affi-Gel Blue agarose column chromatography was also estimated by Sephadex G-100 chromatography (Fig. 4). Coincident profiles for both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity were obtained. A plot of K_{av} versus log (molecular weight) (inset, Fig. 4) gave an

approximate molecular weight of 86 500. The Stokes' radius determined by the procedures of Siegel & Monty (1966) was 4.39 nm. Estimation of the molecular weight of 5.3-S enzyme was also obtained from secondary plots of electrophoretic mobility versus gel concentration (Hedrick & Smith, 1968). At gel concentrations ranging from 4 to 8%, cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity migrated as a single activity band with an approximate M_r of 87 500. A summary of these and other observed physical constants is presented in Table 2. A partial specific volume of $0.725\text{ cm}^3/\text{g}$ (Siegel & Monty, 1966) was assumed in the calculation of the frictional ratio and sedimentation coefficient.

Sucrose-density-gradient analysis of pooled activity from the crude homogenate, $(\text{NH}_4)_2\text{SO}_4$ fraction or DEAE-cellulose stage of purification also resulted in a single symmetrical peak of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity with sedimentation coefficients ranging from 6.5 to 6.6S with an estimated M_r of

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of protein regions of non-denaturing gels. Protein ($285\,\mu\text{g}$) from Affi-Gel Blue-purified fractions was first electrophoresed on 7% non-denaturing acrylamide gels. Slices from the areas corresponding to an R_F of 0.26 and 0.52 were extracted overnight and concentrated by freeze-drying. These concentrated fractions were then electrophoresed on 10% sodium dodecyl sulphate gels. The Figure shows proteins associated with the phosphodiesterase activity region (gel *a*) and the co-purifying protein (gel *b*).

Fig. 4. Gel filtration and estimation of the molecular weight of the 5.3S phosphodiesterase from the Affi-Gel Blue column

The enzyme ($360\,\mu\text{g}$ of protein) from the pooled fractions from Affi-Gel Blue agarose was applied to a column ($2.6\text{ cm} \times 52\text{ cm}$) of Sephadex G-100 and eluted with 25 mM -Tris/HCl, pH 7.4, containing 1 mM - MgCl_2 , 0.5 mM -dithiothreitol and 200 mM -NaCl. Fractions (5.7 ml) were collected and measured for cyclic AMP (●) and cyclic GMP (○) activity at $0.1\,\mu\text{M}$ substrate. The protein standards for molecular-weight determination were phosphorylase *b*, bovine serum albumin, ovalbumin, myoglobin, carbonic anhydrase and ribonuclease. The K_{av} for each of the standards was plotted as a function of the log of molecular weight (inset). The arrow represents the position on the graph of phosphodiesterase.

121000 ± 4900 (11 determinations). Similar values were obtained when soya-bean trypsin inhibitor (10 mg/ml), pepstatin (1 mg/ml) or 0.3 mM-phenylmethanesulphonyl fluoride were included in the homogenization medium as well as the gradient for these fractions. Comparable preparations of the enzyme from freshly isolated homogenates of rat heart gave identical results.

In contrast with the 5.3-S enzyme, the sedimentation coefficient of the enzyme from the DEAE-cellulose column was significantly altered by the addition of EGTA to the sucrose gradients (Fig. 5). The sedimentation coefficients determined in the presence of Ca^{2+} (Fig. 5a) or EGTA (Fig. 5b) were 6.6 S and 5.3 S respectively. Under Ca^{2+} -free conditions calmodulin activity was no longer associated with the enzyme (Fig. 5b) as in the presence of Ca^{2+} (Fig. 5a).

Substrate specificity

At each stage in the purification, phosphodiesterase was monitored to determine the relative rates of hydrolysis of cyclic AMP and cyclic GMP, the extent of EGTA inhibition, and the ability of calmodulin to stimulate enzyme activity. These results are summarized in Table 3. The enzyme purified through the Affi-Gel Blue-stage hydrolysed cyclic AMP and cyclic GMP at approximately the same rate, was not appreciably inhibited by EGTA and exhibited a differential response to calmodulin. The 6.6-S form of the enzyme from the stages before Affi-Gel Blue chromatography hydrolysed cyclic GMP more than 5-fold faster than cyclic AMP.

Response to exogenous calmodulin

Initial experiments indicated that the 5.3-S enzyme was not responsive to added calmodulin even

Table 2. *Physical constants of the 5.3-S phosphodiesterase*

Sedimentation constant	5.3 S
Stokes' radius	4.39 nm
Molecular weight	
Sucrose-density-gradient analysis	86 000
Gel filtration	86 500
Electrophoretic mobility (Hedrick & Smith, 1968)	87 500
Stokes' radius and sedimentation constant (Siegel & Monty, 1966)	95 000
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis	59 000 and 28 000
Fractional ratio (Siegel & Monty, 1966)	1.49

Fig. 5. *Sucrose-density-gradient profile of the 6.6 S phosphodiesterase from the DEAE-cellulose column*

A sample (90.4 μg of protein) of the pooled active fractions from the DEAE-cellulose column was layered on linear sucrose gradients (18–38%) containing 50 mM Tris/HCl, pH 8.0, 5.0 mM-MgCl₂ and 0.5 mM-dithiothreitol and 250 μM-CaCl₂ (a) or 250 μM-EGTA (b). Phosphodiesterase activity in the gradient fractions (0.2 ml) was measured radiometrically for 30 min at 0.1 μM-cyclic GMP (○) and at 0.1 μM-cyclic AMP (●). Calmodulin activity (■) was measured using calmodulin-deficient phosphodiesterase of brain and a 100 μl portion from each fraction. The arrow marks the position of the internal standard (bovine serum albumin). The direction of sedimentation is to the left. The total units of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities recovered in the fractions were 33.5 and 22.4% respectively.

Table 3. *Substrate specificities of cardiac phosphodiesterase at successive stages in the purification process*
Enzyme activity was measured radiometrically at $1.0\mu\text{M}$ -substrate concentration of either cyclic AMP or cyclic GMP under the conditions described below.

Purification stage	Phosphodiesterase activity (nmol/min per mg of protein)								
	Basal*			EGTA†			Calmodulin‡		
	Cyclic AMP	Cyclic GMP	Ratio§	Cyclic AMP	Cyclic GMP	Ratio§	Cyclic AMP	Cyclic GMP	Ratio§
12000 g supernatant	0.150	0.750	5.0	0.045	0.212	4.7	0.184	0.994	5.4
(NH ₄) ₂ SO ₄	0.675	3.98	5.9	0.268	1.26	4.7	0.739	3.98	5.4
DEAE-cellulose	2.21	11.9	5.9	1.00	4.00	4.0	2.92	16.9	5.8
Affi-Gel Blue	118	189	1.6	73.9	110	1.5	147	469	3.2

* Basal activity measured radiometrically in the presence of $250\mu\text{M}$ -CaCl₂.

† Assays carried out in the presence of $250\mu\text{M}$ -EGTA.

‡ Assays carried out in the presence of $4\mu\text{g}$ of calmodulin/ml and $250\mu\text{M}$ -CaCl₂ after EGTA pretreatment.

§ Ratio of cyclic GMP phosphodiesterase/cyclic AMP phosphodiesterase activity.

at very high concentrations (10 and $100\mu\text{g}/\text{ml}$). The addition of 100mM -KCl and 0.1% Lubrol WX did not alter these results. Further, pre-incubation of the enzyme with snake venom ($200\mu\text{g}/\text{ml}$) did not stimulate the hydrolysis of either substrate as expected for a calmodulin-deficient phosphodiesterase. However, it was found that if the enzyme was first pre-incubated with $250\mu\text{M}$ -EGTA and calmodulin added before the addition of Ca²⁺, cyclic GMP phosphodiesterase could be activated by saturating amounts of calmodulin. The hydrolysis of cyclic AMP remained unaffected. These results correlate with previous observations in which the purified enzyme would not bind to calmodulin-Sepharose equilibrated with Ca²⁺-supplemented buffer (results not shown).

The effect of increasing concentrations of exogenous calmodulin on both enzyme activities is presented in Fig. 6. The activity of the enzyme after pre-incubation with EGTA was measured at 1.0 and $100\mu\text{M}$ -cyclic AMP or -cyclic GMP. At low substrate concentration ($1.0\mu\text{M}$), only cyclic GMP phosphodiesterase activity was enhanced by calmodulin, with half-maximal stimulation at 97ng ; cyclic AMP phosphodiesterase activity was not enhanced. At $100\mu\text{M}$ substrate concentration, cyclic AMP phosphodiesterase was stimulated by calmodulin. However, cyclic GMP activity was not stimulated at $100\mu\text{M}$ substrate.

The differential effect of calmodulin on the activity of cyclic AMP and cyclic GMP of the 5.3-S phosphodiesterase measured at $1\mu\text{M}$ prompted an examination of the Ca²⁺ response of the calmodulin-associated 6.6-S enzyme. The Ca²⁺-depleted enzyme was assayed for phosphodiesterase activity in presence of increasing amounts of free Ca²⁺ maintained by a Ca²⁺/EGTA buffer. The results ex-

Fig. 6. *Calmodulin activation of cyclic AMP and cyclic GMP hydrolysis catalysed by the 5.3S phosphodiesterase*

Increasing amounts of calmodulin were added to radiometric assay mixtures containing $1\mu\text{M}$ -cyclic GMP (○), $1\mu\text{M}$ -cyclic AMP (●), $100\mu\text{M}$ -cyclic GMP (□) or $100\mu\text{M}$ -cyclic AMP (■). The reactions were initiated by addition of purified enzyme pretreated with $250\mu\text{M}$ -EGTA. Portions of 144 and 288ng of enzyme protein were added to assay mixtures containing $1\mu\text{M}$ - and $100\mu\text{M}$ -substrate respectively.

pressed as percentage activation of basal enzyme activity are presented in Fig. 7. The addition of Ca^{2+} to the 6.6-S enzyme from DEAE-cellulose activated both cyclic AMP and cyclic GMP hydrolysis in a similar manner, yielding activation constants of 0.76 and $0.45\text{ }\mu\text{M}$ respectively. Hofstee (1952) plots of

Fig. 7. Effect of increasing concentrations of Ca^{2+} on dialysed Ca^{2+} -free phosphodiesterase activity after DEAE-cellulose chromatography

Protein ($90.4\text{ }\mu\text{g/ml}$) was extensively dialysed against 25 mM-Tris/HCl , pH 7.5, containing $0.1\text{ mM-dithiothreitol}$ and 1 mM-MgCl_2 for 24 h. The dialysed enzyme was assayed for hydrolysis of $1\text{ }\mu\text{M-cyclic AMP}$ (●) and cyclic GMP (○) under increasing concentrations of Ca^{2+} in $100\text{ }\mu\text{M-Ca}^{2+}/\text{EGTA}$ buffer. Results are expressed as percentage activation of the basal activity measured in the presence of EGTA.

Ca^{2+} activation did not reveal co-operative interaction. The loss of calmodulin stimulation of cyclic AMP hydrolytic activity was unique to the 5.3-S enzyme.

Kinetic parameters

Kinetic measurements were performed in the concentration range $0.1\text{--}50\text{ }\mu\text{M}$ for both substrates. The kinetic constants for the 5.3-S enzyme before and after storage at -20°C are presented in Table 4. Initial velocity measurements for both substrates gave marked biphasic Hofstee (1952) plots and apparent K_m and V_{max} values were obtained for both phases. When calmodulin ($4\text{ }\mu\text{g/ml}$) was added to the reaction mixtures, normal Michaelis-Menten kinetics were observed for both substrates. After the enzyme was stored at -20°C for 2 months or longer, the enzyme displayed increased specificity for cyclic GMP with little activity toward cyclic AMP when measured in the absence of added calmodulin.

Effect of storage

During this study, it was found that the purified 5.3-S enzyme was transformed after prolonged storage (2–3 months) at -20°C . Sucrose-density-gradient analysis of the aged phosphodiesterase resolved two enzyme activity peaks. A prominent and highly specific cyclic GMP phosphodiesterase sedimented at 6.2S (Fig. 8). This form yielded biphasic kinetic plots with a low K_m of $0.52\text{ }\mu\text{M}$ for cyclic GMP (Table 4) and was also stimulated approx. 2.5-fold by the addition of calmodulin without need of EGTA pretreatment. In addition to the 6.2-S cyclic GMP-specific enzyme, a 4.4-S cyclic nucleotide phosphodiesterase was also evident on sucrose density gradients (Fig. 8).

Discussion

Purification of cardiac phosphodiesterase in the presence of Ca^{2+} resulted in a coincident activity

Table 4. Apparent kinetic constants of the bovine phosphodiesterase isolated from Affi-Gel Blue agarose
Kinetic data were plotted by the method of Hofstee (1952) and each phase of the kinetic plot (I and II) analysed by the method of Eisenthal & Cornish-Bowden (1974).

Condition	Substrate	K_m (μM)		V_{max} . (nmol/min per ml)	
		I	II	I	II
No addition	Cyclic AMP	3.8	71	338	2750
	Cyclic GMP	1.7	5.8	268	568
	Cyclic GMP*	0.52	12.4	28.3	204
Calmodulin† ($4\text{ }\mu\text{g/ml}$) + $250\text{ }\mu\text{M-Ca}^{2+}$	Cyclic AMP	7.7		708	
	Cyclic GMP	2.4		608	

* Aged enzyme (2 months at -20°C).
† Enzyme was pretreated with $250\text{ }\mu\text{M-EGTA}$ and allowed to pre-incubate with calmodulin before addition of Ca^{2+} .

Fig. 8. Demonstration of the transformation of the 5.3-S phosphodiesterase as a result of aging

Protein (2.76 μ g) from an aged portion of Affi-Gel Blue-purified fraction was layered on to 18–38% linear sucrose gradients as described in the Materials and methods section. Fractions (0.2 ml) were collected and analysed for hydrolysis of 0.1 μ M-cyclic GMP (○), cyclic AMP (●) and cyclic AMP with 4 μ g of calmodulin/ml (■). Bovine serum albumin served as an internal protein standard and the arrow marks its position in the gradient.

peak for cyclic AMP and cyclic GMP hydrolysis during all separation procedures including non-denaturing polyacrylamide-gel electrophoresis. The M_r of the enzyme preparations before Affi-Gel Blue chromatography was 121000. This estimate is in agreement with values previously determined for the purified cytosolic phosphodiesterase of bovine cardiac ventricle (Hrapchak & Rasmussen, 1972; Goren & Rosen, 1972). This 6.6-S form of phosphodiesterase appeared to represent the major enzyme species. The production of a 5.3-S enzyme during chromatography on Affi-Gel Blue agarose at high ionic strength was associated with a loss in calmodulin since the activator was no longer detectable on polyacrylamide gels or by direct assay.

The molecular-weight estimates of the 5.3-S enzyme by three independent procedures are in good agreement and yield an approximate value of 89000. The change in sedimentation coefficient from 6.6 S to 5.3 S was consistent with a loss of two subunits of calmodulin with an M_r of 16800 (Watterson *et al.*, 1980).

The 5.3-S enzyme isolated in this study consisted of a 59000 subunit, which is a characteristic of several highly purified enzymes (Klee *et al.*, 1979; La Porte *et al.*, 1979; Morrill *et al.*, 1979; Sharma *et al.*, 1980). However, an approximate molecular weight of 89000, obtained under non-denaturing conditions, would predict a composite dimer for the

purified enzyme. In support of this possibility, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the protein band associated with phosphodiesterase activity from non-denaturing electrophoretic gels yielded protein-staining bands in the region of 28000 and one major band at 59000. However, the staining intensities of these two proteins do not support a 1:1 stoichiometry, and a 28000- M_r protein with phosphodiesterase activity was not detected during this investigation. Interestingly, Pichard & Cheung (1976) have presented evidence of multiple and reversible aggregates of an M_r -22000 enzyme present in liver. Recently, Helfman *et al.* (1981) have characterized an enzyme that resembles a low-molecular-weight cyclic AMP phosphodiesterase (M_r 36500). Tucker *et al.* (1981) and Epstein *et al.* (1978) have described multiple forms of phosphodiesterase that arise due to proteolysis on standing for several days at 4°C. In this study, although rapid and immediate proteolysis cannot be ruled out, care was taken to minimise their action. Cardiac muscle does contain a neutral Ca^{2+} -dependent proteinase (Toyo-oka & Masaki, 1979), which requires Ca^{2+} concentrations above 0.1 mM. However, even at low Ca^{2+} levels, endogenous proteolysis may be more prevalent than in the presence of EGTA. The extent to which endogenous proteolysis may be involved in the present scheme is not known.

The selective response of the catalytic rate of hydrolysis of 1 μ M-cyclic AMP and -cyclic GMP to calmodulin would suggest that distinct active sites for the nucleotides exist on the enzyme. The calmodulin enhancement of the catalytic rate of cyclic AMP hydrolysis at a substrate concentration of 100 μ M would reflect the response of the cyclic GMP-specific site measured at high concentrations of the alternate low-affinity nucleotide. Assays performed at high levels of cyclic nucleotide would preclude observation of the selective action of calmodulin on cyclic GMP hydrolysis. The kinetic results indicated that the catalytic sites may become kinetically equivalent in the presence of calmodulin.

Studies of the enzyme after storage at -20°C provided insight into potential structural rearrangement. On storage, the enzyme transformed into a 6.2-S cyclic GMP-specific form and a 4.4-S cyclic nucleotide phosphodiesterase. This transformation was also marked by a reduction in the K_m for cyclic GMP to the submicromolar range. The cyclic GMP-specific enzyme (6.2 S) may represent a dimer of two 59000- M_r subunits. Studies of the purified brain enzyme indicate a homodimer that is cyclic GMP-specific at micromolar substrate levels (Morrill *et al.*, 1979; Klee *et al.*, 1979). The 4.4-S non-specific phosphodiesterase may include the dissociated monomeric form of the enzyme and possibly a low-molecular-weight phosphodiesterase.

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