Characterization of a Ca^{2+}-Calmodulin-Stimulated Cyclic GMP Phosphodiesterase from Bovine Brain†

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ABSTRACT: A calmodulin-stimulated form of cyclic nucleotide phosphodiesterase from bovine brain has been extensively purified (1000-fold). Its specific activity is approximately 4 μmol min^{-1} (mg of protein)$^{-1}$ when 1 μM cGMP is used as the substrate. This form of calmodulin-sensitive phosphodiesterase activity differs from those purified previously by showing a very low maximum hydrolytic rate for cAMP vs. cGMP. The purification procedure utilizing ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, gel filtration on Sephacryl S-300, isoelectric focusing, and affinity chromatography on calmodulin–Sepharose and Cibacron blue–agarose results in a protein with greater than 80% purity with 1% yield. Kinetics of cGMP and cAMP hydrolysis are linear with K_m values of 5 and 15 μM, respectively. Addition of calcium and calmodulin reduces the apparent K_m for cGMP to 2–3 μM and increases the V_{max} by 10-fold. cAMP hydrolysis shows a similar increase in V_{max} with an apparent doubling of K_m. Both substrates show competitive inhibition with K_i's close to their relative K_m values. Highly purified preparations of the enzyme contain a major protein band of M_r 74 000 that best correlates with enzyme activity. Proteins of M_r 59 000 and M_r 46 000 contaminate some preparations to varying degrees. An apparent molecular weight of 150 000 by gel filtration suggests that the enzyme exists as a dimer of M_r 74 000 subunits. Phosphorylation of the enzyme preparation by cAMP-dependent protein kinase did not alter the kinetic or calmodulin binding properties of the enzyme. Western immunoblot analysis indicated no cross-reactivity between the bovine brain calmodulin-stimulated cGMP phosphodiesterase and the M_r 60 000 high-affinity cAMP phosphodiesterase present in most mammalian tissues.

Multiple forms of cyclic nucleotide phosphodiesterases are distinguishable by their substrate specificity, molecular size, chromatographic behavior, effector molecules, and immunoreactivities (Thompson et al., 1979a,b). A Ca^{2+}-stimulated cyclic nucleotide phosphodiesterase (Type I) has been identified as the predominant form of the enzyme in mammalian brain (Kakiuchi & Tanasaki, 1970). Several laboratories have purified the enzyme from mammalian brain (Klee et al., 1979; Tucker et al., 1981; Kincaid & Vaughan, 1979; Hansen & Beavo, 1982; Sharma, et al., 1980) and bovine heart (Ho et al., 1977; Laporte et al., 1979) by different techniques. Recently, monoclonal antibodies to the bovine heart enzymes have been utilized to isolate forms of the enzyme from bovine brain and heart with similar kinetic properties but having slightly different molecular weights. The enzyme is reported to exist as a dimer of subunit size varying from M_r 61 000 to M_r 57 000, and monomer to dimer conversions during purification have been suggested (Kincaid et al., 1981). These enzymes have been largely characterized with cAMP as the substrate even though the purified enzymes from brain or heart have an order of magnitude higher affinity for cyclic GMP.

Calmodulin, a protein regulator of this enzyme, mediates activation of the enzyme by Ca^{2+} (Kakiuchi et al., 1970; Cheung, 1970, 1971). The x-fold activation of the purified enzyme by calmodulin varies widely (2–50-fold). The degree to which limited proteolysis affects the loss of calmodulin activation of the enzyme remains uncertain (Tucker et al., 1981; Kincaid et al., 1981). Calmodulin increases the V_{max} of the enzyme with cAMP or cGMP as the substrate, but inconsistent effects on K_m values have been noted (Klee et al., 1979; Tucker et al., 1981). The role of stoichiometric levels of phosphorylation of the bovine brain enzyme by cAMP-dependent protein kinase in the regulation of the phosphodiesterase activity remains unclear (Sharma et al., 1980; Van-Eldik & Watterson, 1979). In rat testis, multiple forms of the calmodulin-stimulated phosphodiesterase with differing chromatographic and kinetic properties have been reported (Purvis et al., 1981).

The aim of this study was to investigate the physicochemical, kinetic, and immunological relationship between the calmodulin-stimulated cyclic nucleotide phosphodiesterase (Type I) and the high-affinity cAMP phosphodiesterase (Type IV) also present in all mammalian tissues. This report describes the purification and characterization of a calmodulin-stimulated cyclic GMP phosphodiesterase from bovine brain with kinetic and physicochemical properties distinct from those previously reported. Properties of this enzyme form are compared to those of a high-affinity cyclic AMP phosphodiesterase previously purified in our laboratory (Thompson et al., 1979a,b).

Materials and Methods

Materials. [γ-^{32}P]ATP was purchased from Amersham Radiochemicals. Cyclic [8-^{3}H]cAMP (sp act. 27 Ci/mmol) and cyclic [8-^{3}H]cGMP (sp act. 8 Ci/mmol) were obtained from Schwarz/Mann and New England Nuclear, respectively. Each was purified by anion-exchange chromatography (Thompson et al., 1979a,b) and stored at −20 °C in acidic 50% ethanol. Snake venom (*Ophiophagas hannah*) was purchased from Sigma; cyclic GMP and cyclic AMP were from Boehringer-Mannheim; sodium dodecyl sulfate (SDS) and other chemicals for polyacrylamide gel electrophoresis were from Eastman Kodak. Cyanogen bromide activated Sepharose 4B and Sephacryl S-300 were obtained from Pharmacia. Ultragel AcA 34, ampholine (pH range 3.5–10), and the isoelectric focusing apparatus were from L.K.B. Immobilized Cibacron blue F3GA (cross-linked to agarose) was obtained from Pierce

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Chemical Co., and DEAE-cellulose (DE-52) was purchased from Whatman.

Preparation of Calmodulin–Sepharose. Calmodulin was purified to homogeneity by a modification of the procedure of Yagi et al. (1978) from bovine brain or from rabbit skeletal muscle (Shenolikar et al., 1979). Purified calmodulin was coupled to cyanogen bromide activated Sepharose by a method of March et al. (1974) at 10 mg of protein/mL of swollen gel. The affinity matrix was washed with 6 M urea after use and stored at 4 °C in 0.1 M acetic acid containing 0.2% sodium azide.

Cyclic Nucleotide Phosphodiesterase Assay. cAMP and cGMP phosphodiesterase activities were determined according to Thompson et al. (1979a,b) but using 0.25 μM cAMP and 1.0 μM cGMP as substrates unless otherwise indicated. Ca^{2+}-calmodulin stimulation of the phosphodiesterase activity was determined by comparison of assays in the presence of 100 μM EGTA and assays with Ca^{2+} (10 μM) and calmodulin (50 nM) in the absence of EGTA. One unit of phosphodiesterase activity hydrolyzes 1 μmol of cyclic nucleotide/min at 30 °C. Protein was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

SDS Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 10% (w/v) acrylamide–2.5% (w/v) *N,N'*-methylenebis(acrylamide) resolving gel; a stacking gel of 5% (w/v) acrylamide–1.5% (w/v) *N,N'*-methylenebis(acrylamide) was used.

Purification of Cyclic GMP Phosphodiesterase. Bovine brain was homogenized at low speed for 45 s in a Waring blender in 2.5 volumes of 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.02 mM N^{α}-*p*-tosyl-L-lysyl chloromethyl ketone (TLCK), 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 15 mM 2-mercaptoethanol. The extract was centrifuged at 9000*g* for 30 min, and the resulting supernatant was recentrifuged at 100000*g* for 90 min. Solid ammonium sulfate was added to the second supernatant to obtain 60% saturation. The pH of the solution was adjusted to 7.0 with 0.1 M NH_4OH. After 10 min at 4 °C the precipitate was centrifuged at 15000*g* for 30 min and redissolved ($^1/_{20}$th its original volume) in 40 mM Tris-HCl (pH 8.0) containing 0.02 mM TLCK, 0.1 mM EDTA, and 15 mM 2-mercaptoethanol (buffer A). The dissolved protein was dialyzed against buffer A for 2 h with several buffer changes (approximately 100 volumes total). The dialyzed sample was further desalted on a column of Sephadex G-25 (4.5 × 20 cm) equilibrated in buffer A. All fractions with absorbance at 280 nm in excess of 1.0 OD unit were pooled.

DEAE-cellulose Chromatography. The pooled fractions were applied to a column of DEAE-cellulose (DE-52) equilibrated previously in buffer A and washed until zero absorbance at 280 nm was reached. The column was developed with a linear gradient of 0–0.80 M NaCl in buffer A. The peak fractions of the Ca^{2+}-calmodulin-stimulated phosphodiesterase activity assayed with 1.0 μM cGMP as the substrate were pooled and concentrated either by dialysis against 100% glycerol or in an Amicon concentration cell with a PM-10 membrane.

Gel Filtration on Sephacryl S-300. The concentrated sample was applied to a column of Sephacryl S-300 (2.5 × 90 cm) equilibrated in buffer A and fractionated at a flow rate of 40 mL/h.

Isoelectric Focusing. Pooled fractions of phosphodiesterase activity from the gel filtration step were subjected to preparative isoelectric focusing in an LKB focusing column (440 mL). A glycerol gradient [0–50% (v/v)] containing 1% (w/v) ampholine (pH 3.5–10) was focused for 24 h at 300 V at 4 °C. Peak enzyme fractions were dialyzed for 4 h against buffer A with two changes of 100 volumes each. An additional dialysis bag containing Dowex 1-X8 (chloride form) was used to enhance the extraction of ampholytes from the protein solution.

Affinity Chromatography on Calmodulin–Sepharose. The dialyzed sample was made 0.1 mM Ca^{2+} by the addition of 1 M $CaCl_2$ and applied to a calmodulin–Sepharose affinity column (1.0 × 15 cm) previously equilibrated in buffer A with 0.1 mM $CaCl_2$. The column was washed with the same buffer until the A_{280} dropped below 0.1 OD unit. An intermediate wash consisting of the equilibration buffer containing 200 mM NaCl was utilized prior to elution of enzyme activity with 10 mM EGTA or EDTA in buffer A.

Chromatography on Cibacron Blue (F3GA)–Agarose. The enzyme was purified further by a modification of the method of Morrill et al. (1979) using an immobilized Cibacron blue F3GA–agarose matrix. The enzyme activity peak eluted from calmodulin–Sepharose by EGTA was pooled, made 0.1 mM $CaCl_2$ in excess of the EGTA concentration, and applied to a column of blue–agarose equilibrated 40 mM Tris-HCl (pH 8.0) containing 0.02 mM TLCK, 0.1 mM $CaCl_2$ and 1 mM dithiothreitol (buffer B). Enzyme activity was eluted by inclusion of 1.0 mM EDTA in the equilibration buffer. $CaCl_2$ was added to the phosphodiesterase peak fractions from the first blue–agarose column to give a final concentration of 2 mM before application to a second blue–agarose column equilibrated in buffer B. The column was washed extensively with the buffer B, before elution of the enzyme activity with 40 mM Tris-HCl (pH 8.0) containing 0.02 mM TLCK, 1 mM DTT, 0.1 mM EDTA, and 0.2 mM cGMP. The phosphodiesterase peak was pooled and dialyzed against buffer A containing 50% ethylene glycol (an additional dialysis bag containing Dowex 1-X8 was included to promote removal of cGMP from the sample) and stored at −20 °C.

Kinetic Analysis. The cyclic nucleotide phosphodiesterase activity was measured at various substrate concentrations of cAMP and cGMP in the presence of the labeled substrate (~100 000 cpm/assay) for 10 min at 30 °C in the presence and absence of 10 μM $CaCl_2$ and 50 nM calmodulin. The K_m and V_{max} values were estimated by the kinetic computation program of Cleland (1967). Interaction of cAMP and cGMP as substrates and inhibitors was analyzed by Dixon plots. The nature of the inhibition was confirmed according to Cornish-Bowden (1974). All kinetic determinations were performed on freshly purified preparations.

RESULTS

Purification. The overall purification of a high-affinity cGMP phosphodiesterase is described in Table I. The activity was stimulated by Ca^{2+}-calmodulin. No change in activity was observed in the presence of calcium alone. The enzyme was purified to approximately 80% homogeneity with an overall purification of 1000-fold. The phosphodiesterase activity using 0.25 μM cAMP was also followed during the purification, and the ratio of the rates of hydrolysis of cGMP (1 μM) to cAMP reached a contant value of 80 (ratio corrected for 1 μM substrate concentration) in the final three purification steps, which may suggest the isolation of a single species of enzyme activity. Activation of enyzme activity by calmodulin was maximal following ion-exchange chromatography on DEAE-cellulose but was unstable at the latter stages of purification; a final constant value of 2.5-fold at saturated calmodulin concentrations (50 nM) was obtained upon storage.

Table I: Purification of a Calcium-Calmodulin-Stimulated cGMP Phosphodiesterase from Bovine Brain[a]

	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purificn (x-fold)	CaM activn (x-fold)	cG/cA act. ratio
extract	8869	30.6	0.0035	100	1.0	1.4	4.5
9000*g* sup.	5020	24.5	0.0049	80	1.41	2.3	6.0
100000*g* sup.	4850	24.3	0.0050	79	1.45	3.0	7.0
60% satd ammonium sulfate	1992	23.7	0.012	77	3.45	7.5	10.0
DEAE-cellulose	440	9.06	0.021	30	5.97	16.0	30
Sephacryl S-300	250	6.2	0.025	20	7.19	18.0	44
isoelectric focusing	10.2	2.45	0.239	8.0	69.3	15.0	62
CaM–Sepharose	0.86	1.71	1.99	5.6	576	8.0	85
blue–Sepharose 1	0.15	0.43	2.87	1.4	831	4.8	78
blue–Sepharose 2	0.05	0.18	3.61	0.6	1030	2.5	81

[a] The enzyme activity was assayed in 40 mM Tris-HCl, pH 8.0, at 30 °C by using 1.0 μM cGMP as the substrate (or 0.25 μM cAMP) in the presence of 100 μM EGTA, 10 μM $CaCl_2$ or 10 μM $CaCl_2$, and 50 nM calmodulin in the absence of added EGTA. One unit of enzyme activity was defined as hydrolyzing 1 μmol of the cyclic nucleotide in 1.0 min. The activity ratio for cGMP hydrolysis vs. cAMP hydrolysis was calculated for 1.0 μM substrate concentration.

The K_a for calmodulin activation of the enzyme at all steps after ion-exchange chromatography was consistently 5 nM (data not shown).

Fractionation of cyclic nucleotide phosphodiesterase activity from bovine brain on DEAE-cellulose (Figure 1A) produced two peaks of cGMP hydrolysis, which were not coincident with the two peaks of cAMP hydrolysis observed. One peak of cGMP phosphodiesterase was activated 15–20-fold by the presence of 10 μM $CaCl_2$ and 50 nM calmodulin in the assay. This fractionation indicated that the calmodulin-stimulated phosphodiesterase activity accounted for greater than 90% of the total bovine brain cyclic nucleotide phosphodiesterase activity estimated. The activity peak from DEAE-cellulose was subjected to gel filtration on Sephacryl S-300 (Figure 1B). Two cGMP phosphodiesterase activities were distinguishable with differing degrees of activation by Ca^{2+}-calmodulin. Peak A was activated 5–6-fold by calmodulin whereas peak B was activated 15–20-fold. These peak fractions were pooled and further purified separately.

Peak B contained the major phosphodiesterase activity and was submitted to isoelectric focusing; this yielded a single uniform peak of cGMP hydrolyzing activity with a p*I* of 4.8–5.3 (Figure 1C) and a 10-fold increase in specific activity. Peak A had a slightly higher p*I* of 6.4–6.8 and was stimulated 3–4-fold by calmodulin after isoelectric focusing (data not shown). Kinetic analysis of both phosphodiesterase peaks revealed nearly identical affinities for cGMP and cAMP as the substrate. K_m values for both enzymes were 6.0 μM cGMP and 9.0 μM cAMP in the absence of Ca^{2+} and calmodulin (i.e., 100 μM EGTA) and 3.0 μM cGMP and 30.0 μM cAMP in the presence of 10 μM $CaCl_2$ and 50 nM calmodulin.

Either phosphodiesterase could be adsorbed to the calmodulin–Sepharose affinity matrix in the presence of calcium and eluted with 10 mM EGTA with a 10-fold increase in specific activity but no change in the *x*-fold activation by calmodulin.

Only the peak B phosphodiesterase activity was purified further by affinity chromatography on calmodulin–Sepharose (Figure 1D). A small fraction of the applied activity was eluted with buffer A containing 200 mM NaCl in the presence of 100 μM $CaCl_2$. This activity showed only a 2-fold stimulation of cGMP hydrolysis with Ca^{2+}-calmodulin. Most of the activity was eluted with 10 mM EGTA and resulted in a 10-fold increase in specific activity. The degree of stimulation by calmodulin was 8-fold. In different preparations this was consistently observed and represents a loss of activation from the 10–15-fold observed after DEAE ion-exchange chromatography. This loss did not reflect change in the basal activity assayed in the presence of 100 μM EGTA or altered K_a for calmodulin and occurred in a time-dependent manner (data not shown). This enzyme preparation could be stabilized at a value of 5-fold activation in calmodulin for a period of several weeks when stored at −20 °C in the presence of 50% glycerol.

The pooled activity peak from calmodulin–Sepharose was adjusted to 0.1 mM Ca^{2+} by addition of 1.0 M $CaCl_2$ and then chromatographed on Cibacron blue–agarose as described; this yielded a 25% recovery and a 2-fold increase in specific activity (Figure 2A). The enzyme purified further on blue–agarose (Figure 2B) was relatively less stable and demonstrated only a 2–3-fold activation by calmodulin (as assayed after extensive dialysis to remove cGMP in the sample).

Purity. The purity of the phosphodiesterase preparation was analyzed by SDS electrophoresis on 10% (w/v) polyacrylamide gels. The purified enzyme (5 μg) indicated the presence of a band of M_r 74 000 that accounted for greater than 80% of the total protein (Figure 2C). Electrophoresis of more enzyme demonstrated the presence of additional bands of M_r 59 000 and 46 000. Rechromatography on blue–agarose of various enzyme preparations removed the M_r 46 000 species without significant changes in recoverable activity, suggesting that little if any activity is associated with this protein species. Examination of the proportions of these three species in preparations with differing specific activities indicated that cGMP phosphodiesterase activity best correlates with the M_r 74 000 protein, although the possibility of the M_r 59 000 protein possessing phosphodiesterase activity has not been wholly excluded.[1] Electrophoresis of the purified enzyme preparation on polyacrylamide gels under nondenaturing conditions yielded multiple bands with similar specific activities and may represent aggregated forms of the enzyme (data not shown). Chromatography on hexyl-Sepharose (Couchie et al., 1981), cGMP–Sepharose (Martins et al., 1982), cilostimide–Sepharose (W. J. Thompson, unpublished data), or hydroxylapatite (Klee et al., 1979) failed to completely eliminate the M_r 59 000 protein.

Molecular exclusion on Sephacryl S-300 (K_{av} = 0.35) in the presence of 0.1 mM EDTA indicated that the calmodulin-stimulated cGMP photophodiesterase may chromatograph as a dimer of apparent M_r 145 000 (Stokes radius 35.8 Å). Chromatography on Ultragel AcA34 in the presence of 1 mM EGTA, calibrated with ferritin (M_r 440 000), phosphorylase *b* (M_r 190 000), aldolase (M_r 154 000), BSA (M_r 68 000), and carbonic anhydrase (M_r 26 000) gave a similar apparent M_r of 147 000.

Kinetic Properties. Analysis of freshly prepared phosphodiesterase (800-fold purified from calmodulin–Sepharose)

[1] Data available upon request.

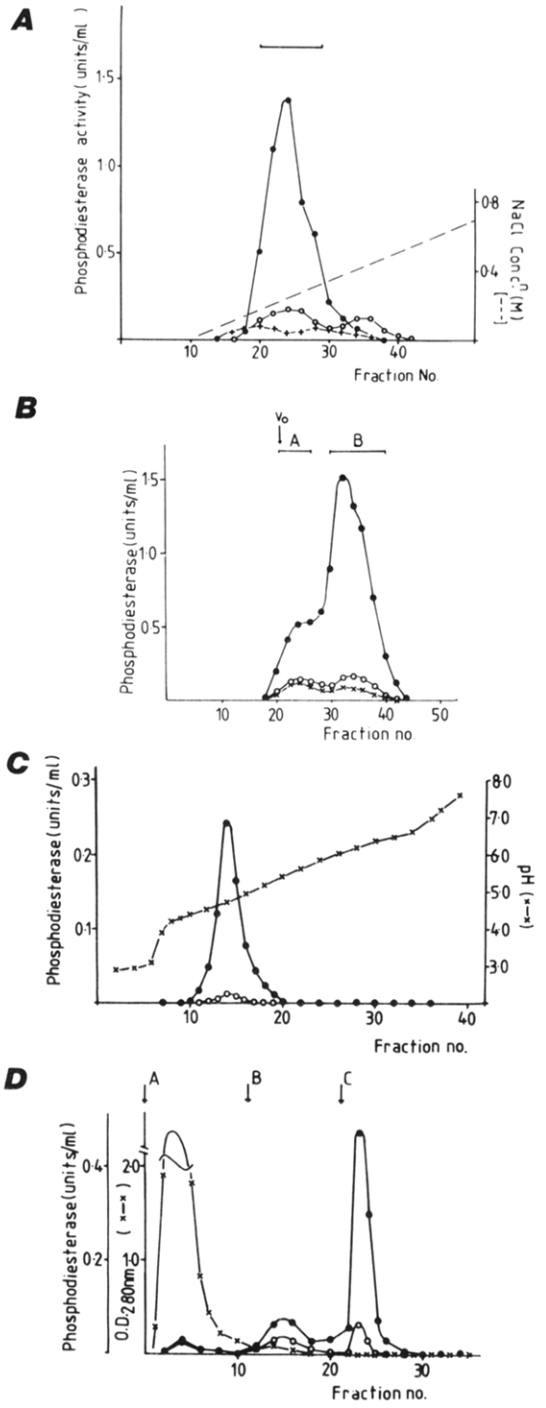

FIGURE 1: Fractionation of soluble cyclic nucleotide phosphodiesterase from bovine brain. (A) Brain extract was chromatographed on DEAE-cellulose (4.5 × 10 cm), eluted at flow rate of 60 mL/h, and 8-mL fractions were collected. The fractions were assayed by using 0.25 μM cAMP (+), 1.0 μM cGMP in the presence of 100 μM EGTA or 10 μM $CaCl_2$ (O), and 1.0 μM cGMP in the presence of 1 μM $CaCl_2$ and calmodulin (50 nM) (●). The bar marks the fractions pooled for further purification. (B) Chromatography on Sephacryl S-300 was carried out as indicated under Materials and Methods, and 5-mL fractions were collected. The fractions were assayed by using 0.25 μM cAMP as the substrate (×). cGMP phosphodiesterase is represented as above. The void volume (V_0) of the column is marked by an arrow. The bar markers represent the fractions pooled and identified as peaks A and B. (C) Isoelectric focusing with a pH gradient 3.5–10.0 as described under Materials and Methods. Enzyme activity is indicated as above. (D) Calmodulin–Sepharose (1.0 × 15 cm) was eluted at flow rate of 20 mL/h, and 3-mL fractions were collected. The phosphodiesterase was applied in the presence of 0.1 mM $CaCl_2$ and washed with buffer A containing 0.1 mM $CaCl_2$ (A). Intermediate wash of buffer A containing 0.1 mM $CaCl_2$ and 200 mM NaCl (B) was applied at fraction 12, as indicated by the arrow. The phosphodiesterase activity was eluted with buffer A containing 10 mM EGTA (C). The enzyme activity was assayed as above.

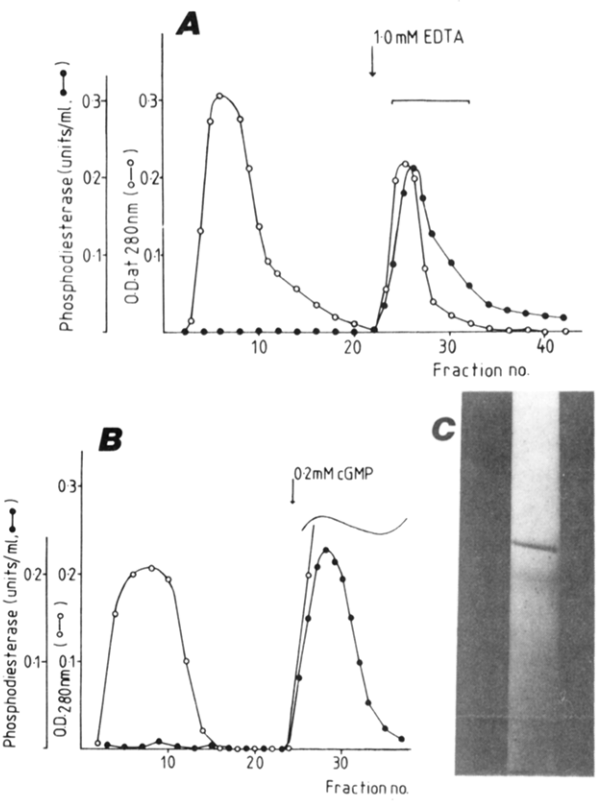

FIGURE 2: Chromatography of cGMP phosphodiesterase activity on Cibacron blue (F3GA)–agarose. (A) The sample was applied to the column (2.0 × 25 cm) in buffer B (as described under Materials and Methods) at a flow rate of 50 mL/h. The phosphodiesterase activity was eluted by buffer B containing 1.0 mM EDTA (↓). The bar marker represents the fractions pooled. (B) The pooled sample from the first Cibacron blue–agarose was applied to a second column of Cibacron blue–agarose equilibrated with buffer B (see Materials and Methods) and eluted with buffer containing 0.2 mM cGMP (↓). The open circles represent absorbance at 280 nm, and the cGMP phosphdiesterase activity, assayed in the presence of 10 μM $CaCl_2$ and 50 nM calmodulin, is represented by the closed circles. The fractions were diluted prior to assaying for enzyme activity, which was corrected for the concentration of cGMP in the elution buffer. (C) The enzyme (5 μg) purified approximately 1000-fold (from the second blue–agarose column) was subjected to electrophoresis on 10% (w/v) polyacrylamide gel in the presence of SDS. The gel was stained for protein with Coomassie blue.

Table II: Effect of Ca^{2+}-Calmodulin on the Kinetics of the Purified Cyclic Nucleotide Phosphodiesterase from Bovine Brain[a]

	100 μM EGTA	10 μM Ca^{2+} + CaM (50 nM)
cGMP hydrolysis		
K_m (μM)	4.76 (±0.5)	2.66 (±0.3)
V_{max} (μmol min^{-1} mg^{-1})	1.09	13.51
K_i (μM) (cAMP)	30.0	40.0
cAMP hydrolysis		
K_m (μM)	13.3 (±5.2)	28.9 (±8.3)
V_{max} (μmol min^{-1} mg^{-1})	0.12	0.89
K_i (μM) (cGMP)	14.0	4.9

indicated linear Michaelis–Menten kinetics for both cGMP and cAMP hydrolysis with a 10-fold greater affinity for cGMP than for cAMP and a 10-fold increase in V_{max} with saturating calcium and calmodulin (Table II). Ca^{2+}-calmodulin increased the V_{max} values for both cyclic nucleotide substrates but reduced the apparent K_m for cGMP 2-fold while increasing the apparent K_m for cAMP by a similar degree. Cyclic GMP hydrolysis was inhibited by cyclic AMP in a simple competitive manner at several substrate concentrations (Figure 3) in the presence and absence of calmodulin. Dixon plots of the data (not shown) showed inhibition of cyclic AMP hydrolysis by

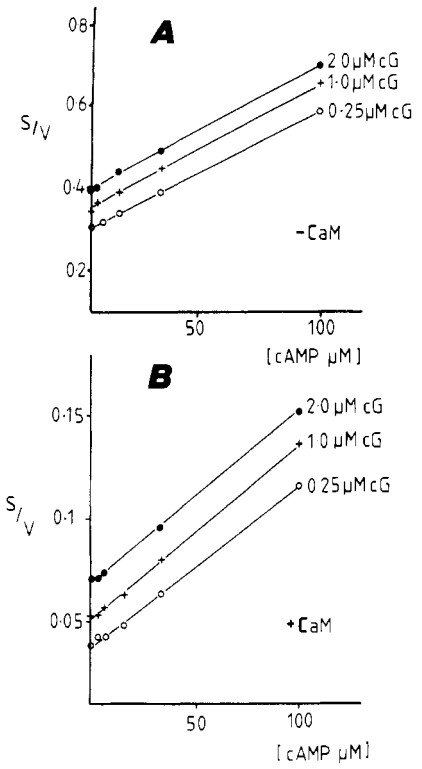

FIGURE 3: Kinetics of the purified cyclic GMP phosphodiesterase in the presence and the absence of calmodulin. The hydrolysis of cGMP at varying concentrations of substrate (0.25, 1.0, and 2.0 μM cGMP) and in the presence of the inhibitor (cAMP) was analyzed according to Cornish-Bowden (1974) in the presence of 100 μM EGTA (panel A) or in the presence of 10 μM $CaCl_2$ and 50 nM calmodulin (panel B).

cyclic GMP ($K_i \simeq 5$ μM) in the presence of calcium and calmodulin. The activation of the phosphodiesterase by Ca^{2+}-calmodulin was completely inhibited by trifluoperazine (100 μM) with no change in the basal activity.

Phosphorylation of Phosphodiesterase. Purified phosphodiesterase preparations (0.3 unit) were maximally phosphorylated within 30 min when incubated with the catalytic subunit of cAMP-dependent protein kinase (1.5 units), purified from rabbit skeletal muscle according to Beavo et al. (1974), 0.2 mM [γ-^{32}P]ATP, and 2 mM $MgCl_2$ at 30 °C. The rate or extent of incorporation was unaffected by the presence or absence of Ca^{2+}-calmodulin with the phosphodiesterase (data not shown). An estimate of the ^{32}P incorporation by fractionation of the labeled proteins on 10% polyacrylamide gel electrophoresis in the presence of SDS indicates 1 mol of phosphate incorporated/mol of M_r 59 000 protein (Figure 4). Minor ^{32}P incorporation was observed in the M_r 46 000 and 40 000 protein bands, but the extent of phosphorylation of the M_r 46 000 and 40 000 could not be accurately estimated. No significant phosphorylation of the major protein species of M_r 74 000 was observed. Phosphorylation of the purified phosphodiesterase did not alter either basal or calmodulin-stimulated enzyme activity using either cAMP or cGMP as the substrate nor did it modify the capacity of the enzyme preparations to bind to calmodulin–Sepharose. The K_a for calmodulin of the phosphorylated enzyme was maintained at 5 nM (using 1.0 μM cGMP as the substrate). The electrophoresis of the phosphorylated enzyme preparation under nondenaturing conditions indicated that the major activity fractions assayed from the gel did not correlate with the ^{32}P-labeled bands observed by autoradiography. However, the recovery of enzyme activity, which correlated with major protein staining bands, was less than 10% (data not shown).

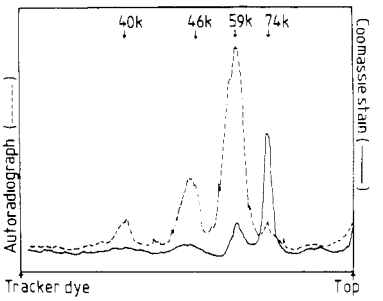

FIGURE 4: Phosphorylation of the cyclic nucleotide phosphodiesterase with cAMP-dependent protein kinase. The cGMP phosphodiesterase (100-fold purified) was incubated with the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP as described under Materials and Methods. The incubation was stopped by heating the sample in the presence of 1% (w/v) SDS and 15 mM 2-mercaptoethanol in a boiling water bath for 5 min. The proteins (15 μg) were analyzed by electrophoresis in 10% (w/v) polyacrylamide gel in the presence of SDS. The gel was stained for protein with Coomassie blue, dried, and autoradiographed to examine ^{32}P incorporation. The diagram represents the comparison of the scans of the autoradiograph (dotted line) and the stained gel (solid line).

Phosphorylation did not significantly alter the multiplicity of bands observed on nondenaturing gels.

Comparative Immunoreactivity of Cyclic AMP and Cyclic GMP PDE. A monospecific sheep antiserum to purified dog kidney cAMP-specific (low K_m) phosphodiesterase (Type IV) of M_r 60 000 (Sarada et al., 1982) was used to test for possible cross-reactivity to Ca^{2+}-calmodulin-stimulated cGMP phosphodiesterase purified from brain by Western immunoblot analysis (Bittner et al., 1980). SDS gel electrophoresis of enzyme preparations at different stages of purification on 10% (w/v) polyacrylamide gel electrophoresis showed several Coomassie staining protein bands. After electrophoretic transfer to nitrocellulose paper and incubation with ^{125}I-labeled antiserum, no detectable levels of the antigen were observed by autoradiography. Fractions analyzed prior to the DEAE-cellulose chromatography step did reveal the presence of a single antigenic species in brain that comigrated with purified low K_m cAMP phosphodiesterase at M_r 60 000 (data not shown). Purified enzyme from dog kidney was detectable at levels approaching 10 ng of protein by the Western immunoblot technique whereas 30 μg of the purified cGMP phosphodiesterase from bovine brain did not cross-react with the ^{125}I-labeled antibody.

DISCUSSION

Several laboratories (Klee et al., 1979; Tucker et al., 1981; Kincaid & Vaughan, 1979; Hansen & Beavo, 1982; Sharma et al., 1980) have developed procedures for isolation and purification of calmodulin-stimulated cyclic nucleotide phosphodiesterase from bovine brain. In general, these studies have shown that the activity resides in a single polypeptide chain of M_r 57 000–63 000 that can exist as a dimer. Purification values of 2000–3500-fold have been reported. Limited kinetic studies point to classification of the enzyme as a "high K_m" phosphodiesterase since it has an apparent K_m for cAMP of 100–200 μM (Klee et al., 1979; Tucker et al., 1981). The enzyme hydrolyzes cGMP with K_m values of 3–9 μM (Tucker et al., 1981; Kincaid & Vaughan, 1979). The V_{max} for cAMP hydrolysis [200–300 μmol min^{-1} (mg of protein)$^{-1}$] may be 10-fold (Morrill et al., 1979) higher than the V_{max} for cGMP hydrolysis [15–50 μmol min^{-1} (mg of protein)$^{-1}$] although similar V_{max} rates have also been reported (Hansen & Beavo, 1982). This enzyme is a Type I phosphodiesterase according to a recently suggested classification system (Strada & Thompson, 1984).

The present report describes the purification of a calmodulin-stimulated cGMP phosphodiesterase from bovine brain. The 4-day purification procedure yielded an enzyme preparation approximately 1000-fold purified, which consisted of greater than 80% of the protein as a M_r 74 000 species with a major contaminant of M_r 59 000 and trace amounts of M_r 46 000 protein observed in the purified preparations. A dimer of M_r 74 000 subunits without associated calmodulin was suggested by gel filtration in the presence of EGTA and by lack of activation of the enzyme activity by calcium ions alone. The purified phosphodiesterase demonstrated a K_m for cGMP of 3.0 μM and cAMP of 30 μM in the presence of Ca^{2+}-calmodulin. Kinetic data indicated that the presence of Ca^{2+} and calmodulin increased the affinity of the enzyme for cGMP while that of cAMP was decreased 2–3-fold. This enzyme preparation indicated a V_{max} value for cGMP of 13.5 μmol min^{-1} (mg of protein)$^{-1}$, similar to those previously reported (Morrill et al., 1979). However, the cGMP phosphodiesterase hydrolyzed cAMP with a V_{max} 2 orders of magnitude lower than previously observed. This suggests that the present purification has yielded a distinct form of the enzyme, which is at least kinetically unrelated to those purified in other laboratories. The investigation of the relationship of the stoichiometry of the M_r 74 000, 59 000, and 46 000 species in preparations with differing degrees of purification indicated that while the M_r 74 000 protein best correlated with the purification, the possibility of the M_r 59 000, which consisted of 10–15% of the protein in the final preparation, possessing some catalytic activity could not be excluded. In agreement with Kincaid et al. (1981), loss in CaM stimulation in the purified preparation can occur in the absence of detectable proteolysis or change in the basal activity. The kinetic properties of the Ca^{2+}-calmodulin-stimulated cGMP phosphodiesterase also indicate that at the physiological concentrations of cGMP and cAMP in the mammalian brain, the enzyme would hydrolyze cGMP at a rate at least 2 orders of magnitude higher than that of cAMP. The polyclonal antibody to a low K_m cAMP phosphodiesterase (Type IV) present in all mammalian tissues (Sarada et al., 1982) did not show immunological cross-reactivity with this cGMP phosphodiesterase even at 2000 times greater concentrations.

The phosphorylation of the enzyme preparation with the catalytic subunit of cAMP-dependent protein kinase suggested stoichiometric incorporation of phosphate into the M_r 59 000 species, with some phosphorylation of M_r 46 000 and 40 000 proteins. The M_r 74 000 protein was not significantly phosphorylated. This could indicate that the M_r 40 000 and 46 000 proteins are derived by proteolysis of the M_r 59 000 species. Tucker et al. (1981) demonstrated the generation of similar M_r fragments from the M_r 63 000 species of the Ca^{2+}-calmodulin-stimulated cyclic nucleotide phosphodiesterase purified from brain due to endogenous proteases. This proteolytic pattern suggests some similarities between the peptides phosphorylated by cAMP-dependent protein kinase and the "high-K_m" enzyme isolated by other laboratories. The stoichiometric phosphorylation of the high-K_m enzyme (M_r 58 000) with cAMP-dependent protein kinase was first reported by Sharma et al. (1980). In agreement with that report, no changes in the kinetic properties of the phosphodiesterase were observed in the present study after phosphorylation of the M_r 59 000 species. The lack of correlation of phosphodiesterase activity with ^{32}P radioactivity on nondenaturing gels suggests the absence of a physical association of M_r 74 000 and the phosphorylated peptides. In addition, it may support the presence of enzyme activity in the M_r 74 000 protein. The low recovery of activity from the gel slices does not permit the elimination of M_r 59 000 protein as a phosphodiesterase. The relationship between the M_r 74 000 species, the M_r 59 000 protein, and the calmodulin-stimulated cyclic nucleotide phosphodiesterase isolated in other laboratories remains unclear. The possibility remains that the limited proteolysis of M_r 74 000 protein to a M_r 59 000 of lower molecular weight fragment might expose a potential site or sites of phosphorylation by cAMP-dependent protein kinase.

The cyclic nucleotide phosphodiesterase partially purified in this study with relatively low yield appears to be "cGMP specific" in comparison to other calmodulin-stimulated cyclic nucleotide phosphodiesterases. This study has also indicated the presence of at least one other species of calmodulin-stimulated phosphodiesterase with apparent M_r 210 000 and pI = 6.4 but possessing some kinetic properties similar to those of the purified enzyme. The existence of other calmodulin-stimulated high-affinity and/or cGMP-specific phosphodiesterases has been indicated in rat testis (Purvis et al., 1981), chicken heart (Andrenyak & Epstein, 1982), and hamster liver (Smoake et al., 1981). This report characterizes some of the properties of one such form from bovine brain. It may exist in relatively lower concentration or may be immunologically different from the major low-affinity phosphodiesterase activity isolated by utilizing a monoclonal antibody from brain by Hansen & Beavo (1982). Further work is required to establish the chemical and immunological relationships of the multiple forms of calmodulin-stimulated cyclic nucleotide phosphodiesterases.

Registry No. cGMP phosphodiesterase, 9068-52-4; cGMP, 7665-99-8; cAMP, 60-92-4.

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Purification and Characterization of the Bifunctional Thymidylate Synthetase-Dihydrofolate Reductase from Methotrexate-Resistant *Leishmania tropica*†

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ABSTRACT: Thymidylate synthetase (TS) and dihydrofolate reductase (DHFR) in *Leishmania tropica* exist as a bifunctional protein. By use of a methotrexate-resistant strain, which overproduces the bifunctional enzyme, the protein was purified 80-fold to apparent homogeneity in two steps. The native protein has an apparent molecular weight of 110000 and consists of two subunits with identical size and charge. Available data indicate that each of the subunits possesses TS and DHFR. The TS of the bifunctional protein forms a covalent 5-fluoro-2′-deoxyuridylate (FdUMP)-(±)-5,10-methylenetetrahydrofolate–enzyme complex in which 2 mol of FdUMP is bound per mole of enzyme. In contrast, titration of DHFR with methotrexate indicated that only 1 mol of the inhibitor is bound per mole of dimeric enzyme. Both TS and DHFR activities of the bifunctional enzyme were inactivated by the sulfhydryl reagent *N*-ethylmaleimide. Substrates of the individual enzymes afforded protection against inactivation, indicating that each enzyme requires at least one cysteine for catalytic activity. Kinetic evidence indicates that most, if not all, of the 7,8-dihydrofolate produced by TS is channeled to DHFR faster than it is released into the medium. Although the mechanism of channeling is unknown, the possibility that the two enzymes share a common folate binding site has been ruled out.

Thymidylate synthetase (TS;[1] EC 2.1.1.45) and dihydrofolate reductase (DHFR; EC 1.5.1.3) catalyze sequential reactions in the de novo synthesis of dTMP. TS catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate; it is unique among enzymes that utilize folate cofactors in that H_4folate is oxidized in the course of the one-carbon transfer reaction. DHFR catalyzes the subsequent NADPH-dependent reduction of the H_2folate produced by TS to regenerate H_4-folate, which serves as a carrier of one-carbon units in a number of metabolic processes. Because blocking either TS or DHFR results in depletion of dTMP and subsequent cessation of DNA synthesis, these enzymes have been studied extensively and exploited as targets for chemotherapeutic agents for a number of diseases.

TS and DHFR are distinct and readily separable in sources as varied as bacteria, bacteriophage, yeast, and vertebrates. Usually, TS is a dimer of identical subunits with a native molecular weight of about 70000 and DHFR is a monomer with a molecular weight of about 20000 [for reviews see Blakley (1984) and Santi & Danenberg (1984)]. In contrast, TS and DHFR have recently been reported to exist as a bi-

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[1] Abbreviations: TS, thymidylate synthetase; DHFR, dihydrofolate reductase; CH_2-H_4folate, (±)-5,10-methylenetetrahydrofolate; H_4folate, (±)-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; MTX, methotrexate; FdUMP, 5-fluoro-2′-deoxyuridylate; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; DTT, dithiothreitol; $NaDodSO_4$-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All other abbreviations are those recommended by IUPAC.

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