Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2788–2792, May 1982
Biochemistry

Purification of two calcium/calmodulin-dependent forms of cyclic nucleotide phosphodiesterase by using conformation-specific monoclonal antibody chromatography

(hybridoma/affinity chromatography/enzyme purification/isozymes)

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Communicated by Earl P. Benditt, January 18, 1982

ABSTRACT **A procedure for nondenaturing immunopurification of bovine calmodulin-dependent 3',5'-cyclic-nucleotide phosphodiesterase (3',5'-cyclic-nucleotide 5'-nucleotidohydrolase, EC 3.1.4.17) is described that utilizes chromatography on a conformation-specific monoclonal antibody column. Hybridomas derived from spleen cells of mice immunized with Ca^{2+}/calmodulin/phosphodiesterase were screened for antiphosphodiesterase antibody production. A stable cell line was established that secretes a monoclonal antibody that binds to the Ca^{2+}/calmodulin/enzyme complex with an approximate K_d of 10^{-9} M. The dissociation constant was increased by two orders of magnitude when calmodulin interaction with the enzyme was inhibited by Ca^{2+} chelation. This differential reactivity was utilized for affinity chromatography of heart and brain phosphodiesterases on monoclonal antibody columns. Highly purified phosphodiesterases were eluted in good yield with buffer containing EGTA. The immunopurified enzymes from heart and brain exhibited specific activities of ≈300 units/mg when assayed at millimolar concentrations of cGMP or cAMP. Calmodulin stimulated both enzymes 10- to 15-fold over basal activity under these conditions. However, analysis of the two preparations by NaDodSO$_4$/polyacrylamide gel electrophoresis revealed an apparent subunit of M_r 61,000 for the brain enzyme, in contrast to the M_r 59,000 cardiac subunit. The observed difference was not an artifact of tissue homogenization because both forms were detected after purification from mixed-tissue homogenates. These results suggest that mild, biospecific elution from a conformation-specific monoclonal antibody column may be a general technique applicable to the rapid isolation of proteins whose antigenic determinants can be altered with specific ligands.**

Multiple forms of cyclic nucleotide phosphodiesterase (3',5'-cyclic-nucleotide 5'-nucleotidohydrolase, EC 3.1.4.17) exist in many mammalian tissues (1). Several reports suggest that one of these forms can be activated by a Ca^{2+}-dependent association with calmodulin (1). Utilization of the Ca^{2+}-dependent binding of this enzyme to a calmodulin-Sepharose affinity column is a basic component of the current methods for purification of this phosphodiesterase (2–4). However, the presence of other calmodulin-binding proteins in these tissues necessitates the use of additional fractionation procedures to obtain purity.

The technique of immunoaffinity chromatography has been useful in the rapid purification of many proteins as well as some enzymes (5). This approach would appear to have widespread application because antibodies can be obtained for most polypeptides. However, the conditions required for elution from antibody affinity columns are generally denaturing and therefore unsuitable for enzyme purification (5). Nonspecific desorption methods are required for elution because of the high avidity, polyclonal nature of antibodies present in conventional antisera. Somewhat milder conditions have been successful for antigen elution from monoclonal antibody columns (6–14). This feature, in addition to the ability of hybridoma technology to provide a virtually unlimited supply of single-site directed antibody from impure antigen, makes immunoaffinity chromatography an attractive alternative to standard purification procedures.

Because many antigenic determinants are thought to be conformational in nature (15, 16), we screened hybridomas for an antibody that would react specifically with the Ca^{2+}/calmodulin/phosphodiesterase complex, yet had little affinity for phosphodiesterase or calmodulin alone. This study reports the use of a monoclonal antibody of this type for the rapid purification of the enzyme from either bovine heart or brain. Some of these data have been reported elsewhere in preliminary form (17, 18).

MATERIALS AND METHODS

Antigen Purification. Calmodulin was purified to homogeneity from bovine blood by using a modification of the fluphenazine-Sepharose method described by Charbonneau and Cormier (19). Homogeneous calmodulin-dependent phosphodiesterase from bovine heart was prepared by a procedure similar to that of Sharma *et al.* (3). The enzyme could be activated 6- to 10-fold by Ca^{2+}/calmodulin.

Preparation of a Hybridoma that Secretes Antiphosphodiesterase Antibody. Immunizations of BALB/c mice, cell fusion of splenocytes with P3-NSI/1-Ag4-1 myeloma cells, hybridoma selection, and cloning procedures were performed as described (18, 20). Antiphosphodiesterase antibody was initially detected by a solid phase radioimmunoassay by using ^{125}I-labeled staphylococcal protein A (18, 20). Growth media from culture cells positive for antibody to Ca^{2+}/calmodulin phosphodiesterase were then tested for their ability to precipitate phosphodiesterase activity from solution by using a staphylococci immunosorbent procedure similar to that described (19). After cloning and subcloning, one cell line (ACAP-1) was established that secreted antibody having a marked requirement for Ca^{2+}/calmodulin for enzyme precipitation. The ACAP-1 antibody was found to be in the IgG_3 class by immunodiffusion analysis with rabbit antisera (Miles) specific for murine μ, γ1, γ2A, γ2B, and γ3 immunoglobulin chains (20).

Immunoprecipitation of Calmodulin-Dependent Phosphodiesterase. The reaction of purified ACAP-1 monoclonal antibody with phosphodiesterase was examined in extracts and pu-

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Abbreviations: P_i/NaCl, phosphate-buffered saline with Ca^{2+} and Mg^{2+} at pH 7.4; PMSF, phenylmethylsulfonyl fluoride; TI buffer, Tris/imidazole/Mg^{2+}/Ca^{2+}/2-mercaptoethanol.

rified preparations by immunoprecipitation (described in Table 1 and Fig. 4). The concentration of enzyme present during the precipitation reaction was based on a specific activity of 400 units/mg for pure calmodulin-deficient phosphodiesterase having a molecular weight of 120,000. Apparent dissociation constants were determined graphically from the antibody concentration (M_r 150,000) at half-maximal enzyme precipitation.

Preparation of ACAP-1 Monoclonal Antibody Affinity Column. Large amounts of ACAP-1 antibody were obtained by injecting 2–20 × 10^6 hybrid cells intraperitoneally into BALB/c mice that had been primed 2 wk previously with a 0.5-ml intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich). Ascites fluid was collected with a syringe 6–20 days after hybridoma injection, centrifuged to remove cells, and stored at −70°C. Homogeneous monoclonal antibody was obtained after chromatography on protein A-Sepharose (Pharmacia) at 4°C (21).

Purified antibody was covalently coupled to Ultrogel AcA 34 (LKB) by using the glutaraldehyde method of Guesdon and Avrameas (22). Glutaraldehyde-activated beads (40 g gravity dried) were washed with 2 vol of 8.7 mM Na_2HPO_4, pH 7.4/83 mM NaCl/8.3 mM KCl/2.5 mM $MgCl_2$/2.8 mM $CaCl_2$ (P_i/NaCl) and incubated with 20 mg of antibody in 40 ml of P_i/NaCl for 24 hr at 4°C. The derivatized resin was washed with 200 ml of 20 mM Tris·HCl, pH 7.5/1 M NaCl and then was incubated with 40 ml of 2 M glycine at pH 7.4 for 16 hr at 4°C to block any remaining reactive sites. The gel was washed and stored in P_i/NaCl containing 3 mM NaN_3. Immunoadsorbants prepared in this manner had a coupling density of 0.25–0.35 mg of protein per gram of wet gel.

Immunopurification of Calmodulin-Dependent Phosphodiesterases. Frozen bovine heart (800 g), brain (100 g), or a mixture of heart and brain (800 g and 100 g, respectively) was homogenized with 40 mM Tris·HCl, pH 8.0/0.5 mM phenylmethylsulfonyl fluoride (PMSF) (2.5 ml/g of tissue) in a Waring Blendor (twice at 15 sec each). The homogenate was centrifuged at 25,000 × g for 30 min and the supernatant was filtered through cheesecloth and glass wool. The extract was adjusted to pH 8.0 with 2 M NaOH and the concentration of PMSF was increased 0.5 mM further. The crude phosphodiesterase was adsorbed batch-wise onto DEAE-cellulose (0.5 ml packed per gram of tissue) for 40 min. The resin was washed with 10 bed vol of 20 mM Tris·HCl, pH 7.5/1 mM imidazole/1 mM $MgCl_2$/100 μM $CaCl_2$/15 mM 2-mercaptoethanol (TI buffer) containing 0.5 M NaCl and the phosphodiesterase was eluted with TI buffer/0.4 M NaCl. Pooled fractions of high activity were diluted with 2 vol of TI buffer and applied to the antibody column (1.5 × 9 cm). The column was washed with 80 ml of TI buffer/0.15 M NaCl and then washed with 65 ml of TI buffer/0.05 M NaCl. Phosphodiesterase was eluted with the latter wash buffer containing 2 mM EGTA at a flow rate of 10 ml/hr. Further enzyme elution was accomplished with 2 M $MgCl_2$. All operations were carried out at 4°C. The electrophoretic analysis of purified antigens and antibody was performed on $NaDodSO_4$/7.5–15% polyacrylamide gels under reducing conditions (23). Purified calmodulin and IgG_3 concentrations were determined spectrophotometrically by using an $A^{1\%}_{276}$ of 1.8 (24) and an $A^{1\%}_{280}$ of 14.5 (25), respectively. The Coomassie G-250 dye-binding assay was employed for other proteins with bovine serum albumin as the standard (26).

Phosphodiesterase Assay. Cyclic nucleotide phosphodiesterase activity was measured by the two-step radioisotope procedure as described (27) except that DEAE-Sephadex A-25 was substituted for Dowex as the ion-exchange resin. The enzyme was routinely determined by incubating samples for 10 min at 30°C in a reaction mixture that contained 20 mM Tris·HCl/20 mM imidazole·HCl at pH 8.0, 3 mM $MgCl_2$, bovine serum albumin at 0.2 mg/ml, 1 mM ^{3}H-labeled cyclic nucleotide (≈60,000 cpm), and either 0.2 mM $CaCl_2$ and calmodulin at 4 μg/ml or 2 mM EGTA. One unit of enzyme catalyzes the hydrolysis of 1 μmol of cAMP per min in the presence of 1 mM cAMP and saturating Ca^{2+}/calmodulin.

RESULTS

Ca^{2+}-Dependent Immunoprecipitation of Phosphodiesterase Activity. Table 1 shows that ACAP-1 monoclonal antibody precipitates either homogeneous or crude phosphodiesterase obtained from heart tissue. The precipitation was dependent on Ca^{2+}/calmodulin because EGTA inhibited the binding reaction about 10-fold. The precipitated activity was quantitatively recovered in the immunosorbant pellet, indicating that the antibody is not inhibitory. The Ca^{2+}-dependent reaction was specific for calmodulin because Ca^{2+} alone did not enhance the precipitation of calmodulin-deficient enzyme (data not shown). The Ca^{2+}/calmodulin sensitivity of the interaction suggested that the ACAP-1 antibody was specific for the calmodulin-dependent isozyme of phosphodiesterase. To examine the specificity more directly, the two major forms of cardiac phosphodiesterase were resolved by DEAE-cellulose chromatography. Fig. 1 shows that the antibody only recognized the Ca^{2+}/calmodulin-sensitive enzyme peak.

Utilization of ACAP-1 Monoclonal Antibody for Purification of Bovine Heart and Brain Phosphodiesterases. Phosphodiesterase activity in extracts of heart or brain was concentrated on DEAE-cellulose before application to an antibody affinity column. The concentrated fraction that contained endogenous calmodulin was diluted to reduce the NaCl concentration to <0.20 M and applied to an antibody-Ultrogel column. The chromatographic profile of brain phosphodiesterase shown in Fig. 2 was also typical for the heart enzyme. The activity that passed through the column represented ≈30% of the total units applied, but it was only slightly sensitive to EGTA inhibition (1.4-fold). Approximately 50% of the adsorbed activity was eluted with buffer containing EGTA. The remaining activity could be desorbed in a less purified state with the addition of

Table 1. Effect of EGTA on precipitation of cardiac phosphodiesterase by ACAP-1 antibody

Precipitation condition	Phosphodiesterase activity,* units × 10^3	
	Supernatant	Pellet
Pure phosphodiesterase		
Boiled ACAP-1 Ca^{2+}/calmodulin	10.4	0
Boiled ACAP-1 EGTA	9.80	0
ACAP-1 Ca^{2+}/calmodulin	0.53	8.80
ACAP-1 EGTA	8.70	1.01
Crude extract phosphodiesterase		
Boiled ACAP-1 Ca^{2+}/calmodulin	12.9 (4.78)	ND
Boiled ACAP-1 EGTA	12.3 (4.69)	ND
ACAP-1 Ca^{2+}/calmodulin	4.82 (3.45)	ND
ACAP-1 EGTA	11.5 (4.56)	ND

Phosphodiesterase and purified ACAP-1 antibody (1.3 μg) were incubated on ice for 2 hr with 0.2 ml of a 10% suspension of heat-killed, formalin-treated *Staphylococcus aureus* Cowan 1 strain bacteria in the presence of Ca^{2+}/calmodulin or EGTA assay buffer. The suspension (0.5 ml total) was centrifuged at 1,000 × g for 1 min at 4°C and the supernatant was separated from the pellet. ND, not determined.

* Aliquots of the supernatant or resuspended pellet were assayed for phosphodiesterase activity by using Ca^{2+}/calmodulin and 0.5 mM cAMP. Activity in parentheses was determined in the presence of EGTA.

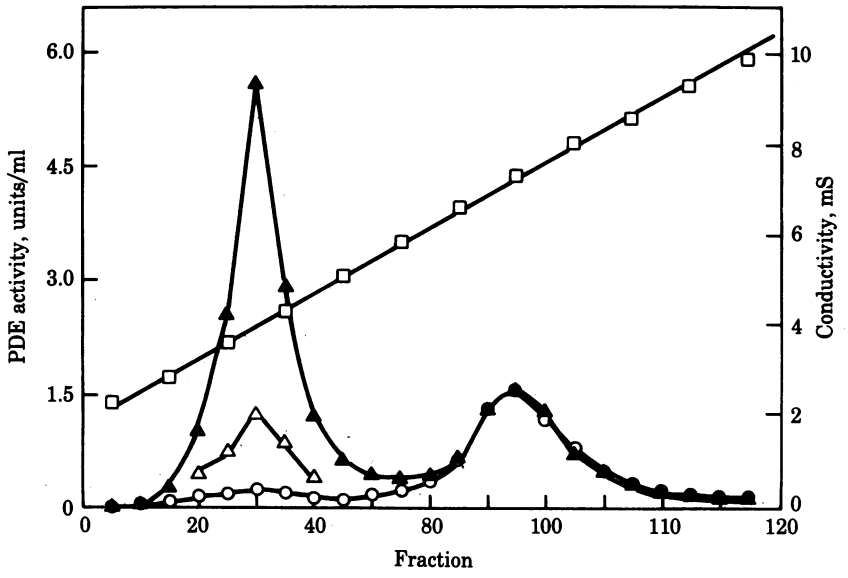

FIG. 1. ACAP-1 antibody interaction with cardiac cAMP phosphodiesterases. Extract from 1 kg of heart tissue was adjusted to 3 mM EGTA and applied to DEAE-cellulose. The column (5 × 26 cm) was washed with Ca^{2+}-free TI buffer/0.05 M NaCl containing 0.1 mM EGTA and was eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. Antibody precipitations were carried out as in Table 1 by using 0.02-ml column fractions and 5 μg of antibody. Phosphodiesterase (PDE) activity was measured by using 0.5 mM cAMP as described. ▲, Activity in the presence of Ca^{2+}/calmodulin; △, activity in the presence of EGTA; ○, residual activity in the presence of Ca^{2+}/calmodulin after antibody precipitation; □, conductivity.

2 M $MgCl_2$ to the elution buffer (data not shown). This procedure resulted in substantial purification of either the heart or brain enzyme. Table 2 indicates that the two enzymes were of high specific activity and could be activated 10- to 15-fold by calmodulin. Either preparation was capable of hydrolyzing cGMP at the same rate as cAMP at millimolar substrate concentrations (data not shown). The antibody affinity column provided a single-step purification of up to 3,000-fold from the DEAE-cellulose fraction. The high degree of electrophoretic purity of these enzymes is shown in Fig. 3.

Comparison of the enzymes isolated from brain and heart tissue revealed a distinct difference (≈2,000) in apparent molecular weight. The cardiac enzyme comigrated with the M_r 59,000 enzyme prepared from heart by more conventional procedures (data not shown). In contrast, the immunopurified brain enzyme migrated above the M_r 60,000 catalase standard. Fig. 3 also demonstrates that both enzyme forms can be isolated after homogenization of a brain/heart tissue mixture. The two tissues were combined such that each would contribute approximately equal amounts of phosphodiesterase to the extract. Because roughly equal amounts of each form were present after purification, the observed difference was not a tissue-specific artifact introduced during or subsequent to homogenization.

Affinity of Immunopurified Phosphodiesterase for ACAP-1 Antibody. Catalytic amounts of the immunopurified phosphodiesterases were precipitated with varying amounts of ACAP-1 antibody and *Staphylococcus aureus* to quantitate the dependence of the interaction on Ca^{2+}/calmodulin. Analysis of the

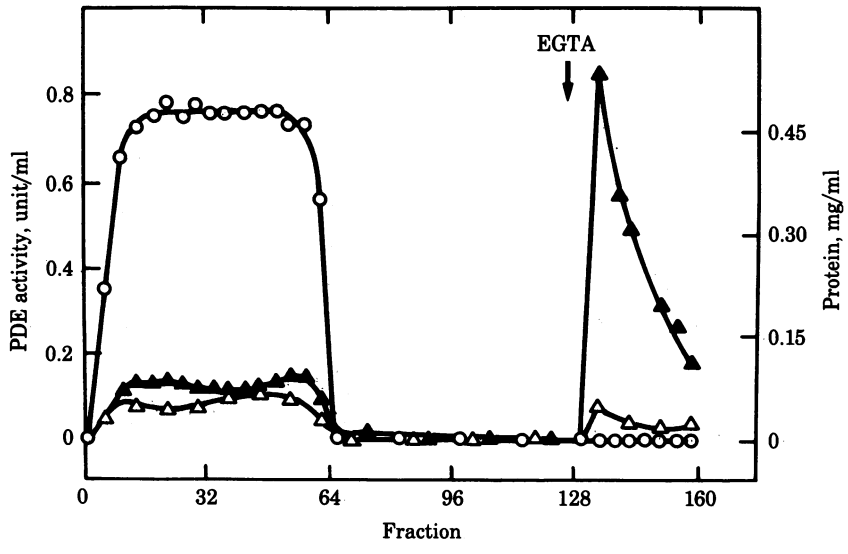

FIG. 2. Immunoaffinity chromatography of bovine brain calmodulin-dependent phosphodiesterase. Partially purified extract was applied to an ACAP-1 antibody column, washed with Ca^{2+}-containing buffer, and eluted with the addition of EGTA as described; 1.6-ml fractions were collected. Phosphodiesterase (PDE) activity was assayed by using 1 mM cAMP in the presence of Ca^{2+}/calmodulin (▲) or EGTA (△). ○, Protein concentration.

Table 2. Purification of heart and brain calmodulin-dependent phosphodiesterase

Fraction	Total protein, mg	Total activity,* units	Specific activity, units/mg	Yield, %	Purification, fold
		Heart†			
Extract	16,000	240 (100)	0.015	100	1.0
DEAE-cellulose	1,200	102 (26)	0.085	43	5.7
Antibody-Ultrogel	0.080	22 (2.1)	270	9.0	18,000
		Brain‡			
Extract	1,100	130 (46)	0.12	100	1.0
DEAE-cellulose	350	67 (11)	0.20	51	1.6
Antibody-Ultrogel	0.071	21 (1.4)	300	16	2,400

* Activity was determined by using 1 mM cAMP and saturating Ca^{2+}/calmodulin as described. Activity in parentheses was determined in the presence of EGTA.
† Data are based on 800 g of bovine ventricle.
‡ Data are based on 100 g of bovine cerebral cortex.

data shown in Fig. 4 revealed antibody dissociation constants in the presence of Ca^{2+}/calmodulin of 1.4×10^{-9} M and 2.0×10^{-9} M for the heart and brain enzymes, respectively. Precipitation in the presence of EGTA resulted in a 100-fold shift in the apparent dissociation constants of both isozymes to a value of $\approx 2.0 \times 10^{-7}$ M.

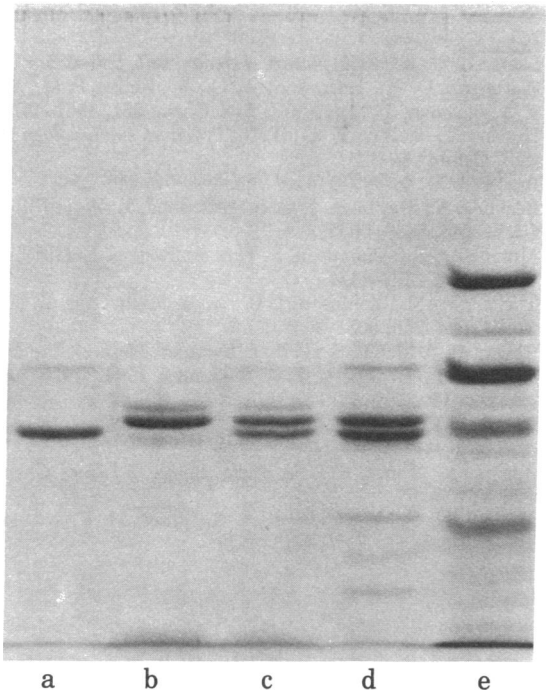

FIG. 3. Differentiation of purified heart and brain phosphodiesterases by $NaDodSO_4$/7.5% polyacrylamide gel electrophoresis. Calmodulin-dependent phosphodiesterases were isolated by immunoaffinity chromatography of DEAE-cellulose fractions obtained from extracts of heart, brain, and a mixture of heart and brain as described. Lanes: a, purified heart phosphodiesterase (2 μg); b, purified brain phosphodiesterase (2 μg); c, heart phosphodiesterase (1 μg) and brain phosphodiesterase (1 μg); d, phosphodiesterase purified from a mixture of heart and brain (4 μg); e, M_r standards: phosphorylase b from rabbit muscle, 95,000; bovine serum albumin, 68,000; bovine liver catalase, 60,000; ovalbumin, 43,000.

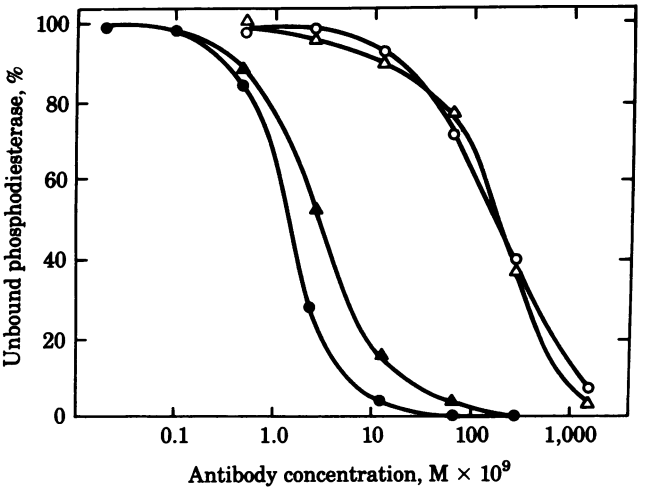

FIG. 4. Effect of Ca^{2+}/calmodulin on heart and brain affinity for ACAP-1 antibody. Immunopurified enzymes from heart or brain ($2.0–2.5 \times 10^{-3}$ unit) were incubated with varying amounts of antibody (0–120 μg) as described in Table 1. Activity remaining in the supernatant after precipitation was determined by using 20 μM cGMP in the presence of saturating Ca^{2+}/calmodulin. Precipitation of cardiac enzyme in the presence of Ca^{2+}/calmodulin (●) or EGTA (○). Precipitation of brain enzyme in Ca^{2+}/calmodulin buffer (▲) or EGTA buffer (△).

DISCUSSION

Protein antigenic determinants have been divided into two general categories termed "sequential" and "conformational" (15). The antigenic determinant recognized by the antiphosphodiesterase antibody appears to be of the conformational type because its formation is markedly inhibited when the enzyme is not bound to Ca^{2+}/calmodulin (Table 1, Figs. 2 and 4). Although it is possible that a portion of the calmodulin molecule contributes to the antigenic determinant, this situation is unlikely because calmodulin does not interact with the antibody directly (data not shown) and a large excess of calmodulin does not prevent phosphodiesterase precipitation (Table 1, Fig. 4). In addition, other calmodulin-binding proteins do not copurify with the cyclic nucleotide phosphodiesterase as shown in Fig. 3. Whatever the nature of the phosphodiesterase determinant, it is clear that the putative conformational shift between a catalytically inactive and active state induced by calmodulin (1) can be utilized for nondenaturing immunopurification of the enzyme.

The phosphodiesterases purified from heart and brain were different in apparent subunit molecular weight (Fig. 3), but they had similar catalytic and immunological properties (Tables 1 and 2, Fig. 4). Conceivably, a conversion of the M_r 61,000 brain-type isozyme to a M_r 59,000 form may have resulted from a cardiac-specific covalent modification such as proteolysis. However, if such a process occurred, it must have been complete before cell disruption because both forms were isolated from mixed-tissue homogenates (Fig. 3). Most previous reports have not suggested a tissue-specific molecular difference between the heart and brain enzymes (3, 4, 28). However, a recent report has suggested that multiple forms can be isolated from brain tissue depending on the presence or absence of protease inhibitors (29). The relationship of these forms to the enzymes we have isolated in the presence of PMSF is unknown.

The immunopurification technique described for the cal-

modulin-dependent phosphodiesterase system has significant advantages over conventional procedures published to date. Contamination with other calmodulin-binding proteins is often a problem in phosphodiesterase purifications that rely on calmodulin-Sepharose affinity chromatography (3, 4). The antibody affinity column does not appear to recognize such proteins and therefore obviates the necessity of additional separation steps. In addition to the ease and rapidity of purification, the enzymes are never exposed to buffers containing detergents, high salt concentrations, or extremes of pH. Therefore, the method seems ideal for comparison of the structure and activity of the calmodulin-dependent phosphodiesterases in different tissues.

Antibody affinity columns are known to adsorb proteins nonspecifically and these usually remain as contaminants when nonspecific desorption methods are employed (30). Antigen purification from cell-free extracts by using a monoclonal antibody column often results in actin contamination (9, 11). The $MgCl_2$ elution of cardiac phosphodiesterase from the ACAP-1 antibody column contains four major polypeptides other than the phosphodiesterase: M_r 200,000, 45,000, 36,000, and 30,000 (data not shown). Therefore, the use of EGTA for phosphodiesterase elution not only has the advantage of being nondenaturing, but it also increases the degree of purification. Screening for a conformation-specific monoclonal antibody may therefore be a viable approach to the rapid, nondenaturing purification of many other enzymes and labile proteins.

Other examples of chromatographic purification based on the conformation-specificity of immunoreactions include isolations of Ca^{2+}-dependent polyclonal antibodies. This has been accomplished for antibodies directed against a random copolymer $(Glu^{60}Ala^{40})_n$ (31), two acidic polysaccharides (32), and prothrombin (33). A recent report of β-adrenergic receptor isolation by using a monoclonal antibody column may be another example of conformation-specific immunoaffinity chromatography (13). In this procedure, elution of the receptor was accomplished with the β-adrenergic antagonist, propranolol. Aside from their use in purification, conformation-specific monoclonal antibodies may also be excellent probes for quantitating a given antigenic conformation (e.g., activation state) present *in vivo* or *in vitro* (34). The ACAP-1 monoclonal antibody may have promise in this regard for under certain conditions it appears to react with and stabilize only the calmodulin-activated phosphodiesterase (Fig. 1, Table 1).

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