ticularly suited to cardiovascular investigation. Antibodies to the purified protein may not only contribute insight to renin biosynthesis and the nature of renin precursors but also may provide a highly specific renin antagonist in physiologic experiments.

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References

Cohen, S., Taylor, J. M., Murakami, K., Michelakis, A. M., & Inagami, T. (1972) *Biochemistry 11*, 4286.
Corvol, P., Devaux, C., Ito, T., Sicard, P., Ducloux, J., & Menard, J. (1977) *Circ. Res. 41*, 616.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.
Haas, E., Lamfrom, H., & Goldblatt, H. (1954) *Arch. Biochem. Biophys. 48*, 256.
Haber, E., Koerner, T., Page, L. B., Kliman, B., & Purnode, A. (1969) *J. Clin. Endocrinol. Metab. 29*, 1349.
Inagami, T., & Murakami, K. (1977) *J. Biol. Chem. 252*, 2978.
Lowry, D. M., Rosenbrough, N. J., Farr, A. I., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Marciniszyn, J., Hartsuck, J. A., & Tang, J. (1976) *J. Biol. Chem. 251*, 7088.
Murakami, K., & Inagami, T. (1975) *Biochem. Biophys. Res. Commun. 62*, 757.
Reinharz, A., & Roth, M. (1969) *Eur. J. Biochem. 7*, 334.
Rodbard, D., & Chrambach, A. (1971) *Anal. Biochem. 40*, 95.
Rubin, J. (1972) *Scand. J. Clin. Lab. Invest. 29*, 51.
Udenfried, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., &. Weigele, M. (1972) *Science 178*, 871.
Vestergerg, O., & Svensson, N. H. (1966) *Acta Chem. Scand. 20*, 820.
Weber, K., & Osborn, N. (1969) *J. Biol. Chem. 244*, 4406.

# Purification and Characterization of High-Affinity Cyclic Adenosine Monophosphate Phosphodiesterase from Dog Kidney†

W. Joseph Thompson,* Paul M. Epstein,‡ and Samuel J. Strada

ABSTRACT: High-affinity cyclic adenosine monophosphate (cAMP) phosphodiesterase was purified from dog kidney homogenate to apparent homogeneity. The high-affinity form of the enzyme system was separated from the bulk of low-affinity cAMP–cGMP phosphodiesterase by preparative DEAE-cellulose chromatography and further purified by hydroxylapatite chromatography, DEAE-cellulose rechromatography, and Sephacryl S-200 gel filtration in a stabilizing buffer system. Criteria of purity were sodium dodecyl sulfate–acrylamide gel electrophoresis, sedimentation velocity, sedimentation equilibrium, and gel filtration. The enzyme shows no evidence of subunit structure, is an acidic protein as determined by amino acid composition and isoelectric focusing (p$I$ = 4.8), and has a molecular weight of 48 000–60 600. Determinations of diffusion, sedimentation, and frictional coefficients and Stokes' radii suggest that an ellipsoid shape of the protein apparent in a low-polarity medium is more stable than a more spheroidal shape apparent in a high-polarity medium. The purified enzyme displays Michaelis–Menten kinetic behavior for cAMP and cGMP hydrolysis with relative affinities of 2.2 and 312 $\mu$M, respectively. cAMP hydrolysis is not affected by known activator or inhibitor proteins. We conclude from these studies that this purified high-affinity enzyme form is the basic catalytic subunit of mammalian cyclic nucleotide phosphodiesterases.

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) catalyzes the hydrolysis of cAMP and cGMP, the only known catabolic mechanism for these important regulatory nucleotides. Both kinetic and physical criteria have supported the existence of multiple molecular forms of this enzyme system in a wide variety of tissues. High- and low-affinity enzyme forms have been identified that differ in size, substrate specificity, and modulation by effectors such as cAMP or cGMP, a calcium-dependent regulator (calmodulin), modulator binding proteins (inhibitor), cell–cell interactions, proteases, hormones, drugs, and genetic influences [for reviews, see Appleman & Terasaki (1975), Wells & Hardman (1977), Strada & Thompson (1978), and Thompson & Strada (1978)].

Purification of mammalian high- and low-affinity cyclic nucleotide phosphodiesterases has not previously been achieved, and thus the biochemical elements of this enzyme system were not known. This stands in contrast to effectors of one of the lower affinity forms of the enzyme, calcium-dependent regulator or calmodulin and inhibitor proteins, which have been purified to homogeneity (Lin et al., 1974; Watterson et al., 1976; Vandermeers et al., 1977; Klee & Krinks, 1978; Wang & Desai, 1977; Jarrett & Penniston, 1978; Sharma et al., 1978). Some nonmammalian sources of the enzyme have been extensively purified, but these appear to differ markedly in their kinetic and physical properties (Rosen, 1970; Morishima, 1974; Fujimoto et al., 1974; Miki et al., 1975) from mammalian enzyme forms.

Partial purification of the low-affinity form of the enzyme has been reported from heart (Butcher & Sutherland, 1962;

† From the Department of Pharmacology, The University of Texas Medical School at Houston, Houston, Texas 77030. *Received March 9, 1979.* Supported by U.S. Public Health Service Grant GM 21361 and the Pulaski County Cancer Society, Pharmaceutical Manufacturer's Association, and Rosalie B. Hite Foundation.

‡ Present address: Department of Pharmacology, University of Connecticut, Farmington, CT.

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Goren & Rosen, 1972; Ho et al., 1977), brain (Watterson & Vanaman, 1976; Miyake et al., 1977; Brostrom & Wolff, 1976), adrenal (Siegel & Egrie, 1977), lung (Davis & Kuo, 1977), pancreas (Terai et al., 1976; Schubart et al., 1974), and liver (Thompson & Appleman, 1971a; Moss et al., 1978) tissues and the high-affinity enzyme from skeletal muscle (Huang & Kemp, 1971), lung (Bergstrand et al., 1978), platelets (Hidaka & Asano, 1976), and liver (Loten et al., 1978) with limited criteria of purity and property characterization.

In these studies, we report the purification to apparent homogeneity of a high-affinity cAMP phosphodiesterase form of the enzyme system from dog kidney and characterization of the purified enzyme. These results are discussed in reference to practical purification problems and to hypotheses concerning the relationship of low- and high-affinity enzyme forms.

## Experimental Procedures

*Materials.* [8-$^3$H]cAMP (sp act. 20 Ci/mmol) and [8-$^3$H]-cGMP (sp act. 17.2 Ci/mmol) were purchased from Schwarz/Mann and New England Nuclear, respectively, purified by using Dowex 1-X2 (200–400 mesh), and stored at −20 °C in acidic 50% ethanol. [2-$^3$H]Adenosine (sp act. 8 Ci/mmol) and [8-$^3$H]guanosine (sp act. 17 Ci/mmol) were from New England Nuclear and were purified by thin-layer chromatography. The following materials were purchased as indicated: snake (*Ophiophagus hannah*) venom, cAMP, cGMP, PMSF,[1] TLCK, Mes, DTT, Brij 35, Bicine (Sigma); magnesium chloride, ethylene glycol, sodium fluoride, sucrose (Fisher); Tris, Hepes (Calbiochem); benzamidine (Eastman Kodak); ampholytes (LKB); and Fluram (Hoffmann-La Roche). Dowex 1-X2 (200–400 mesh) (Bio-Rad), DE-11 (Whatman), Sephacryl S-200 (SF), and DEAE-Sephacel (Pharmacia) were prepared according to manufacturer's specifications. All reagents and stains used for electrophoresis were from Bio-Rad or Eastman Kodak and were of electrophoretic grade purity. Enzymes and proteins used for column or gradient calibration, their sources, and their properties [$s_{20,w}$ (S), $D_{20,w}$ ($\times 10^{-7}$ cm$^2$/s), molecular weight] were as follows: bovine serum albumin monomer (4.2–4.7, 5.9–6.0, 66 000), human serum transferrin (5.1–6.1, 5.0–5.3, 68 000–74 000) (Miles); egg white ovalbumin (3.55–3.66, 7.6–7.8, 43 000–45 000), bovine pancreas chymotrypsinogen A (2.5–2.6, 9.5–10.2, 22 000–25 000), liver catalase (11.3, 4.1, 232 000–250 000, 57 500–60 000), rabbit muscle pyruvate kinase (237 000, 57 200) (Worthington); horse heart cytochrome *c* (1.7–2.5, 11.4–13.3, 12 300–15 000), beef heart lactic dehydrogenase (7.0–7.4, 4.5–5.5, 35 000) (Sigma); milk lactoperoxidase (5.4, 6.0, 77 500) (ICN); human serum γ-globulin (6.6–7.3, 3.7–4.0, 160 000) (Schwarz/Mann); and rabbit muscle aldolase (7.4–8.9, 4.6–5.2, 149 000–150 000, 40 000) (Nutritional Biochemical).

*Cyclic Nucleotide Phosphodiesterase Activity Analysis and Protein Determination.* cAMP and cGMP phosphodiesterase activities were measured by the two-step radioisotope procedure of Thompson & Appleman (1971b) modified to minimize nonspecific reaction product binding and high ionic strength "blank" interference (Epstein et al., 1977; Thompson et al., 1979). Unless otherwise indicated enzyme dilutions for activity analyses were in 40 mM Tris-HCl (pH 8.0)–BSA (0.5 mg/mL). Assays were initiated no more than 10 min postdilution. Reaction mixtures (0.4 mL) contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, [$^3$H]cAMP or [$^3$H]cGMP ($\simeq$100 000 cpm), unlabeled cAMP or cGMP (0.25–100 μM), and 30 μg of bovine serum albumin. Reactions were incubated at 30 °C for 10 min and terminated by boiling for 45 s. The background radioactivity which was routinely below 1% remained below 2% with up to 0.5 M sodium acetate or phosphate but increased substantially with equivalent concentrations of sodium chloride or sulfate. Therefore, acetate or phosphate salts were used throughout these studies as anion displacement for ion-exchange chromatography.

For stabilization of cAMP phosphodiesterase activity, complex elution and storage buffers were employed in these studies (vide infra). These buffers interfered with the modified Lowry (Schacterle & Pollack, 1973), Bradford (1976), or fluorescamine (Udenfriend et al., 1972) protein assays. Consequently, protein values are reported as intrinsic fluorescence with excitation at 280 nm and emission at 340 nm (Udenfriend, 1969). Critical values were verified by the above procedures after perchloric acid precipitation, extensive dialysis against $H_2O$, and/or standard curves of bovine serum albumin in the appropriate buffer. Ion-exchange and gel filtration column profiles were monitored by using absorbance at 280 nm and by intrinsic fluorescence.

*Tissue Homogenization and Preparative Centrifugation.* Twenty frozen dog kidneys (Pelfreeze, Inc.) were chopped into small pieces and homogenized in a Waring blender in a cold room (640 g wet weight) in 4 L of 10 mM Tris-HCl (pH 8.0)–20 μM TLCK–50 mM NaF for 1 min (4×), allowing for cooling between each time. The homogenate was centrifuged at 9000*g* for 30 min with a Beckman JA 10 rotor (4 °C). The resultant supernatant was adjusted to pH 6.0 with acetic acid, filtered through glass wool, and centrifuged at 105000*g* for 90 min with a Beckman Type 30 or 35 rotor. Approximately 20% of the homogenate cGMP phosphodiesterase activity and 35% of the cAMP phosphodiesterase activity are lost in the pellet at this centrifugation step. Ninety percent of the pelleted cGMP phosphodiesterase activity is recoverable from these particulate fractions following rehomogenization with a polytron in the hypotonic buffer, whereas only 30% of the pelleted cAMP phosphodiesterase activity is recoverable; this procedure was not routinely applied for preparative purposes. The phosphodiesterase activity of the 105000*g* supernatant is subject to a variety of proteolytic and other influences and is not stable in the homogenization buffer system; it is necessary to apply this supernatant immediately to the first DEAE-cellulose column which is equilibrated in the enzyme-stabilizing buffer system to minimize these factors; this buffer is used subsequently throughout the purification.

*Purification of Dog Kidney High-Affinity cAMP Phosphodiesterase. Step 1.* The high-affinity form of cAMP phosphodiesterase was purified by first separating it from the bulk of low-affinity cAMP phosphodiesterase–cGMP phosphodiesterase activities by using preparative DEAE-cellulose anion-exchange chromatography. Whatman DE-11 cellulose was washed, defined, equilibrated in 5 mM Mes–30% ethylene glycol–1 mM DTT–5% glucose (w/v)–15 mM sodium acetate–50 mM NaF–20 μM TLCK (pH 6.5, EG buffer), and packed to a 1-L bed volume in a 5 × 60 cm column. By use of ascending flow, 4 L of the 105000*g* kidney supernatant (36 g of protein) was applied to the cellulose and the column was washed with 2–4 bed volumes of the equilibrating buffer. The

[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-α-*p*-tosyl-*l*-lysine chloromethyl ketone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; Bicine, *N*,*N*-bis(2-hydroxyethyl)-glycine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Fluram, 4-phenylspiro[furan-2(3*H*),1′-phthalon]-3,3′-dione; DEAE, diethylaminoethyl; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; NaDodSO$_4$, sodium dodecyl sulfate; Nbs$_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

column was then washed with EG buffer containing 150 mM sodium acetate (pH 6.5) until the eluting protein was below 0.1% of that applied. cGMP phosphodiesterase (peak I) and cAMP phosphodiesterase (peak II) were eluted with a 3600-mL curvilinear gradient of sodium acetate in EG buffer (pH 6.5) from 150 to 1200 mM using a nine-chambered Buchler Varigrad gradient maker. An additional 2 L of the concentrated buffer was required to complete the enzyme elution. Gradient fractions of 13 mL were collected at a rate of 2.3 mL/min.

*Step 2.* Fractions from 3.8 to 5.0 L (750 mg of protein) of the first DEAE-cellulose column were pooled and applied to granulated hydroxylapatite (GHA; Mazin et al., 1974) equilibrated in EG buffer with 10 mM potassium phosphate (pH 7.0) substituted for sodium acetate and Mes. GHA was packed to 300 mL in a 2.5 × 60 cm column. By use of ascending flow, the column was washed with 2 bed volumes of equilibration buffer, and cAMP phosphodiesterase activity was eluted by using 10–350 mM potassium phosphate gradient (pH 7.0; 900 mL) fractions at 1.2 mL/min.

*Step 3.* DEAE-Sephacel (Pharmacia) was equilibrated in EG buffer (pH 6.5) and packed to a 10-mL bed volume in a 1.2 × 30 cm column. One hundred and sixty milliliters (~120 mg of protein) of the GHA activity peak was applied to the column which was then washed with 100 mL of EG buffer containing 250 mM sodium acetate (pH 6.5). High-affinity cAMP phosphodiesterase activity was eluted with a linear, 50-mL, 250–1500 mM sodium acetate gradient (EG buffer; pH 6.5) in 1.2-mL fractions at 0.3 mL/min.

*Step 4.* Sephacryl S-200 (SF) was packed to a 370-mL bed volume in a 2.6 × 90 cm column. This column was equilibrated and the enzyme eluted in EG buffer (pH 6.5)–0.5 M sodium acetate at 1.0 mL/min. Fractions 14–24 (10 mL; ~20 mg of protein) of the second DEAE-cellulose column were pooled and applied to the column. Four-milliliter fractions were collected. The column was calibrated by using standards as indicated, and $K_{av}$ was calculated as $(V_e - V_0)/(V_t - V_0)$ with Blue Dextran 2000 as an indicator of $V_0$ and $^3H_2O$ as an indicator of $V_t$.

*Analyses by Ultracentrifugation.* Sedimentation and diffusion coefficients for purified cAMP phosphodiesterase were determined by comparison with standard proteins using linear sucrose gradients (5–20%) buffered with 50 mM $K_2PO_4$ (pH 6.8). Gradients of 5 mL were prepared with a gradient maker in polyallomer tubes; enzyme or standards stored in EG buffer were diluted 1:40 in 50 mM $K_2PO_4$ (pH 6.8), and 0.2-mL aliquots were layered on the gradients which were then centrifuged at 49 000 rpm in a Beckman SW 50.1 rotor for 14 h and fractionated by piercing the bottom of the tubes. Six standards were used, two each in three adjacent tubes.

Sedimentation velocity experiments were used as an analysis of purity and to determine the sedimentation coefficient of the protein in undiluted EG buffer. The homogeneity of the purified enzyme was determined by using 3 mg/mL enzyme in EG buffer in a double sector cell with EG buffer as the reference. Centrifugation was at 60000*g* at 20 °C with a Beckman Model E ultracentrifuge. Due to the extreme curvature of the reference buffer refractive index, the sedimentation coefficient was determined by comparison with the rate of bovine serum albumin (4 mg/mL) in EG buffer. Fourteen comparative migration measurements were determined during the course of centrifugation (48 h) in order to establish the relative $s_{20,w}$ for cAMP phosphodiesterase.

High-speed sedimentation equilibrium centrifugation was used to test the homogeneity of the enzyme and to determine the mass of the enzyme. These studies were conducted in collaboration with Dr. Philip Cohen, Department of Biochemistry, University of Dundee, Dundee, Scotland. The protein concentration was 0.5 mg/mL, and the buffer was 100 mM $K_2PO_4$–30% ethylene glycol–15 mM 2-mercaptoethanol (pH 7.0). Centrifugation was in a Beckman Model E at 26000 rpm (10 °C). The partial specific volume of cAMP phosphodiesterase was calculated from amino acid composition data according to Cohn & Edsall (1943) as 0.732 at 20 °C. A temperature correction factor $d\bar{v}/dT = 0.0005$ mL/(g K) was applied.

*Analyses by Electrophoresis.* The subunit molecular weight and column fraction purity were analyzed by using 10% acrylamide slab gels in 0.1% sodium dodecyl sulfate with the discontinuous buffer system of Laemmli (1970). The running buffer was 0.1% $NaDodSO_4$–3% glycine–50 mM Tris-HCl (pH 8.3). The enzyme, column fraction, or standard proteins (1–100 μg) were precipitated with 10% trichloroacetic acid (4 °C), centrifuged at 10000*g* for 15 min, dissolved in 10 μL of 50% glycerol–10 mM DTT–0.1% bromophenol blue–1% $NaDodSO_4$, and boiled for 3 min after adjustment of the pH to 7.0. Electrophoresis was for ~3 h at 140 V. Gels were washed overnight with 25% 2-propanol to remove $NaDodSO_4$, stained with 0.25% Coomassie Brilliant Blue R 250–0.1 M $AlCl_3$–1% Triton X-100–45% methanol–10% acetic acid (Hegenaver et al., 1977) for 45 min at 50 °C, and destained with successive 45% methanol–10% acetic acid and 7.5% methanol–10% acetic acid washes. Alternatively, cAMP phosphodiesterase was stained according to the procedure of Green et al. (1973) for acidic proteins. After removal of $NaDodSO_4$, the gels were stained with 0.005% "Stains All"–5% dimethylformamide–25% 2-propanol–15 mM Tris-HCl (pH 8.8) for at least 24 h in the dark. The stained gels were stored in the dark in diluted staining solution (10%) to avoid bleaching.

cAMP phosphodiesterase activity was determined by using similar 10% acrylamide gels, except $NaDodSO_4$ was omitted and 30% ethylene glycol was added to the polymerization buffer. The running buffer was 5 mM Tris-HCl–30% glycine (pH 8.3). Seventy-five microliters (25 μg) of purified enzyme was dialyzed against ethylene glycol in a 400-μL Lucite dialysis chamber for 2 h (eight changes). Electrophoresis of three 15-μL samples was at 250 V for 3 h; one sample was stained with Coomassie Blue and one with Stains All, and one was assayed for cAMP phosphodiesterase activity. For activity analysis, gels were sliced in 1-mm slices and each was incubated with 300000 cpm [$^3$H]cAMP–5 mM $MgCl_2$–5 mM 2-mercaptoethanol–40 mM Tris-HCl (pH 8.0) for 3 days at 4 °C or overnight at room temperature. Snake venom was added, and the [$^3$H]adenosine product was determined as above.

Isoelectric focusing was accomplished with 100 μg of enzyme by using a linear density gradient of 10–40% glycerol in an LKB 8101 column at 2–4 °C. The anode solution was 0.5 N $H_2SO_4$ in 65% glycerol (bottom), and the cathode solution was 1% (w/v) ethanolamine. Constant voltage (560 V) was applied for 65 h with an LKB 3371 dc power supply. The amperage drop was 3.4–0.4 Å during focusing. Eighty fractions (1.5 mL) were collected and the pH and enzyme activity in each fraction determined.

*Amino Acid Analysis.* cAMP phosphodiesterase was dialyzed against water, precipitated with 10% sulfosalicylic acid, resuspended in $H_2O$, and lyophilized in hydrolysis tubes. Duplicate 25-μg samples were hydrolyzed in vacuo in 6 N HCl for 24, 48, and 72 h at 100 °C. The samples were processed with a Durrum 400 amino acid analyzer with a three-buffer

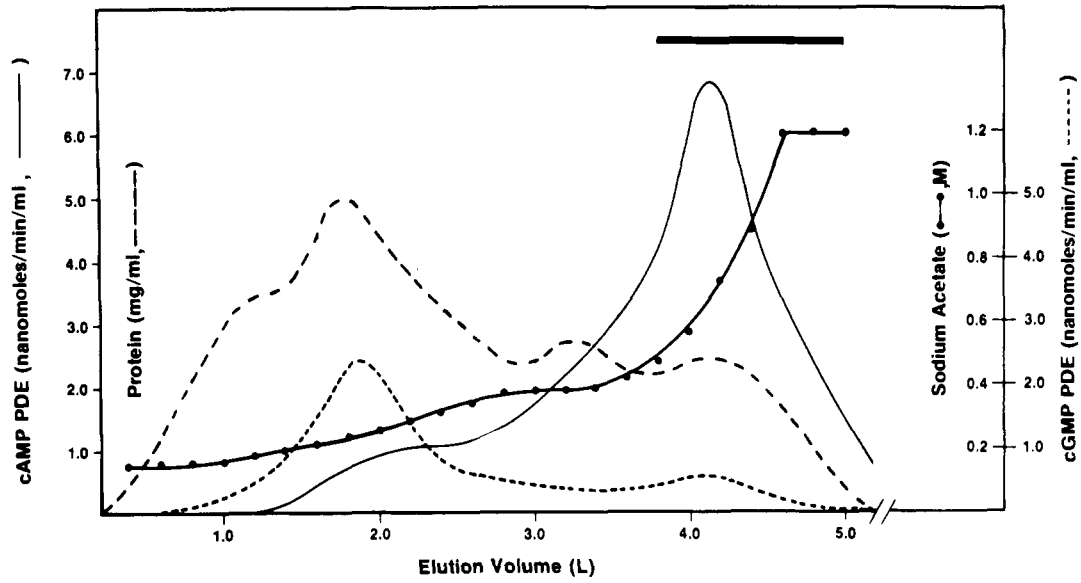


FIGURE 1: Preparative DEAE-cellulose chromatography of the dog kidney 105000*g* supernatant. 36 g of protein was applied to 1 L of DEAE-cellulose equilibrated in EG buffer (pH 6.5). cAMP and cGMP phosphodiesterases were eluted with 5 L of 150–1200 mM sodium acetate in EG buffer (pH 6.5) at 2.3 mL/min in 13-mL fractions. See Experimental Procedures for the details of chromatography. An aliquot of the gradient fraction was diluted in 40 mM Tris-HCl (pH 8.0)–bovine serum albumin (0.5 mg/mL), and within 10 min of dilution activities were determined by using 0.25 μM cAMP and 1 μM cGMP as substrates. Profiles are the composite of four separate preparations. Protein values were determined by absorbance at 280 nm and intrinsic fluorescence (280–340 nm). Fractions from 3.8 to 5.0 L were pooled for further purification.

elution system. Cysteine was determined by titration of the $NaDodSO_4$-denatured enzyme with $Nbs_2$ (Ellman, 1959). Tryptophan was determined as a ratio to tyrosine according to Bencze & Schmid (1957), and the spectrum was verified by using an equivalent mixture of L-tryptophan and L-tyrosine under the same conditions.

## Results

*Purification of High-Affinity cAMP Phosphodiesterase.* High-affinity cAMP phosphodiesterase was separated from the bulk of lower affinity enzyme activity found in the 100000*g* supernatant of dog kidney by preparative DEAE-cellulose chromatography. Kidney tissue was chosen because it has a relatively high specific activity compared to other tissues and because it has a relatively high percentage of soluble vs. particulate high-affinity enzyme activity (Van Inwegen et al., 1977). Approximately 75% of the activity measured with 0.25 μM cAMP as substrate was recovered in the 100000*g* supernatant when the tissue was homogenized in a hypotonic buffer (Table I). These data confirm earlier studies of enzyme distribution in other species (Dousa & Rychlik, 1970; Gulyassy et al., 1975; Jard & Bernard, 1970; Filburn & Sacktor, 1976). The homogenization buffer also included NaF and TLCK in an attempt to minimize the effects of cellular proteases and the possible covalent modification mechanism (Sakai et al., 1978; Terai et al., 1976; Epstein et al., 1978). Although we have not studied these agents in a systematic fashion, their deletion from the homogenization buffer with the preparative procedure yielded lower recoveries and poorer resolution of enzyme forms. Inclusions of benzamidine and PMSF as additional protease inhibitors appeared to have no effect over that of TLCK and NaF alone. Homogenization in isotonic sucrose instead of the hypotonic medium showed a high amount of high-affinity enzyme in the 9000*g* and 100000*g* pellets, and only a minor portion of this activity was released by further washes and rehomogenization with a polytron, observations similar to those of Filburn et al. (1977) in rabbit and Van Inwegen et al. (1977) in rat kidney. The 100000*g* supernatant should be applied to the DEAE column as soon as possible to prevent "aging" or an activating effect upon storage of the supernatant at 4 °C. Acidification of the 9000*g* supernatant had no effect on enzyme activity, but did aid in protein precipitation in the 100000*g* centrifugation step and prevented nonphosphodiesterase protein binding to DEAE-cellulose.

Table I: Purification of Dog Kidney High-Affinity cAMP Phosphodiesterase

| | total act.[a] (nmol/min) | total protein[b] (mg) | sp act. [nmol/(min mg)] | recovery (%) | *x*-fold purifcn |
|---|---|---|---|---|---|
| homogenate | 15 700 | 85 000 | 0.18 | 100 | 1.0 |
| 9000*g* supernatant | 13 100 | 47 100 | 0.28 | 83 | 1.5 |
| 9000*g* pellet | 1 200 | | | 8 | |
| 100000*g* supernatant | 11 800 | 36 500 | 0.32 | 75 | 1.8 |
| DEAE-cellulose[c] | | | | | |
| peak I | 1 900 | 3 500 | 0.54 | 12 | 3.0 |
| peak II | 3 600 | 740 | 4.86 | 23 | 27 |
| GHA of peak II activity peak | 1 026 | 120 | 8.55 | 6.5 | 48 |
| DEAE-cellulose[d] activity peak | 830 | 37 | 22.4 | 5.3 | 124 |

[a] cAMP phosphodiesterase activities were determined by using 0.25 μM substrate. [b] Protein was quantitated by using intrinsic fluorescence (280–340 nm). [c] First column. [d] Second column.

cGMP phosphodiesterase eluted from DEAE-cellulose at ~250 mM sodium acetate (peak I) and cAMP phosphodiesterase at 650 mM sodium acetate (peak II) under these conditions (Figure 1). A curvilinear gradient of sodium acetate in EG buffer was used to allow optimal elution of cGMP phosphodiesterase. Approximately 93% recovery of applied high-affinity cAMP activity was achieved; however, only 31% of this activity was pooled for further purification. A 15-fold increase in specific activity was observed. Fifty-seven percent of the applied activity measured with 0.25 μM cAMP substrate either coeluted at low ionic strength with cGMP

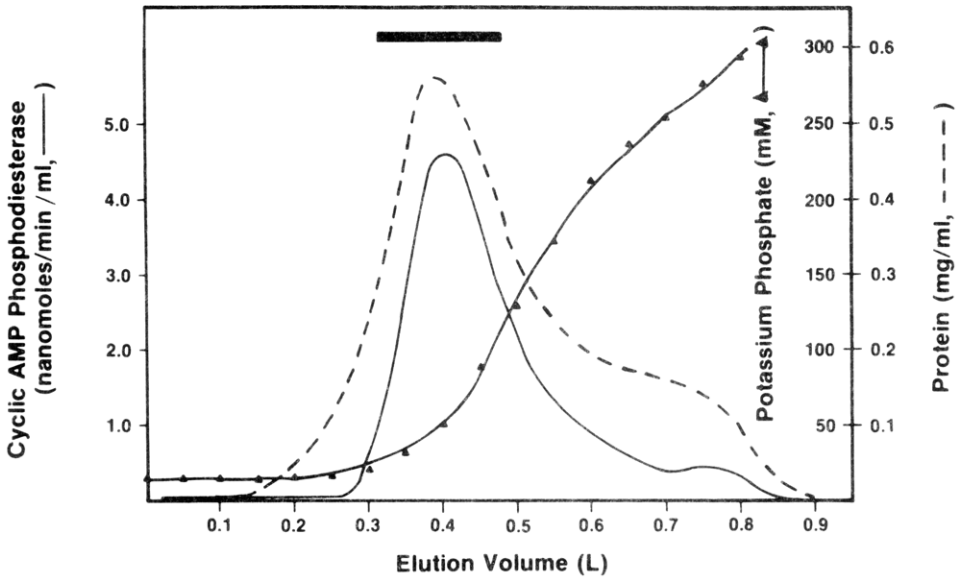


FIGURE 2: Granulated hydroxylapatite chromatography of cAMP phosphodiesterase from DEAE-cellulose (DEAE peak II). Pooled fractions from the second peak of cAMP phosphodiesterase activity obtained from the preparative DEAE-cellulose chromatography (750 mg of protein; 1.2 L) were applied to 300 mL of GHA equilibrated in 10 mM potassium phosphate–EG buffer (pH 7.0). cAMP phosphodiesterase was eluted with a 10–350 mM gradient of potassium phosphate (900 mL; pH 7.0 in EG buffer) in 9-mL fractions at 1.2 mL/min. Activity and protein values were determined as in Figure 1. Profiles are the composite of four preparations.

phosphodiesterase activity or was not pooled because of a high percentage of contaminating protein. The EG buffer system, when used at an equilibrating pH of 6.5, prevented nearly 80% of the 100000*g* supernatant protein from binding and stabilized the various isolated, partially purified, and purified forms of phosphodiesterases for at least several months.

Granulated hydroxylapatite was used as the next purification step in order to avoid dilution, dialysis, or ultrafiltration of peak II phosphodiesterase, procedures that led to inactivation of activity and compromised preparative level procedures (Figure 2). Approximately 45% of the protein applied did not bind to GHA, which nearly accounts for the 1.8-fold purification obtained by this procedure (Table I). A steep potassium phosphate gradient was required to obtain adequate activity recovery and to eliminate some contaminants that proceeded the activity peak, factors that precluded a batch procedure. Forty-three percent recovery was obtained from the DEAE peak II activity applied, and 28% of this activity was pooled for subsequent purification.

The GHA-fractionated enzyme was then rechromatographed on either DEAE-cellulose or DEAE-Sephacel. Elution of the enzyme with a linear sodium acetate gradient (in EG buffer) achieved a 100% recovery of the GHA pooled activity applied (Figure 3). Seventy-seven percent had the final specific activity of the purified enzyme [22 nmol/(min mg) at 0.25 $\mu$M cAMP substrate]. The protein and activity profiles were nearly coincident, except in the low salt portion of the gradient where most of the remaining contaminants were eluted (Figure 3), as shown by $NaDodSO_4$–acrylamide gels.

cAMP phosphodiesterase was freed of remaining contaminants by gel filtration using Sephacryl S-200 (SF) (Figure 4). Activity recovery with Sephacryl S-200 was 80% of that applied, and the major protein peak was coincident with the activity peak. Besides a small percentage of higher molecular weight protein, the major contaminant removed was nearly pure CDR, a contaminant that results from its overlap with peak II enzyme on the first DEAE column. As revealed by $NaDodSO_4$–10% acrylamide slab gels (Figure 4), the peak of activity corresponds to the peak of the dense protein band of the gels. Upon overloading the gels, a minor "doublet" of smaller molecular weight was seen in some but not all preparations. This trace contaminant can be removed by rechromatography on a small GHA column. These minor protein bands have no activity and probably occur during homogenization. Experiments with several protease inhibitors included in the $NaDodSO_4$ boiling and trichloroacetic acid ($Cl_3AcOH$) precipitation procedures indicate that the trace contaminants are not produced by $Cl_3AcOH$ participation or during $NaDodSO_4$

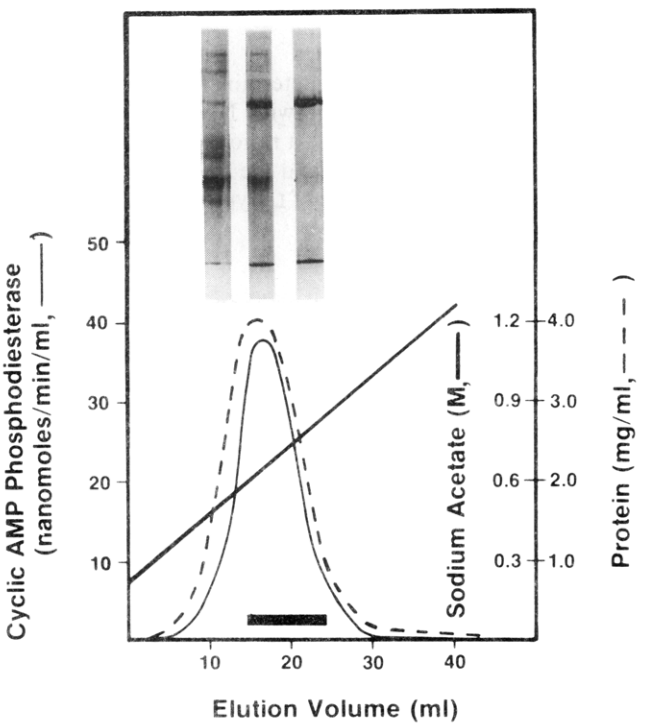


FIGURE 3: DEAE-cellulose (-Sephacel) rechromatography of GHA DEAE peak II cAMP phosphodiesterase. 160 mL (120 mg of protein) of the GHA peak activity was applied to 10 mL of DEAE-Sephacel equilibrated in EG buffer. Activity was eluted with a linear 250–1500 mM sodium acetate gradient as indicated, and activity and protein values were determined as in Figure 1. Profiles are the composite of four separate preparations. 10% $NaDodSO_4$–acrylamide slab gel analyses are shown for 30 $\mu$g of the 13-, 19-, and 24-mL fractions. Gels shown were cut for presentation from an analysis of the entire profile.

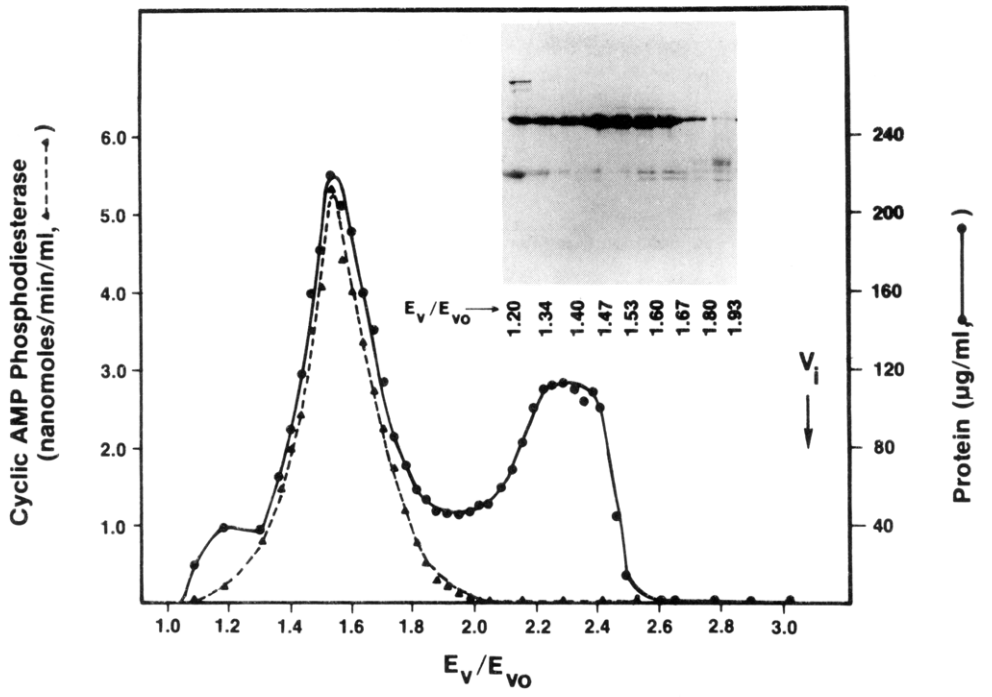


FIGURE 4: Sephacryl S-200 (SF) gel filtration of cAMP phosphodiesterase activity from DEAE-cellulose rechromatography. Fractions from the 14th through the 24th milliliter (20 mg of protein) from the previous purification step were pooled and applied to a 370-mL Sephacryl S-200 (SF) column equilibrated in EG buffer (pH 6.5)–0.5 M sodium acetate. Activities and proteins were determined upon elution as in Figure 1. Equal volumes of the fractions indicated were precipitated with 10% $Cl_3AcOH$ and analyzed by 10% $NaDodSO_4$–acrylamide slab gel electrophoresis as described under Experimental Procedures.

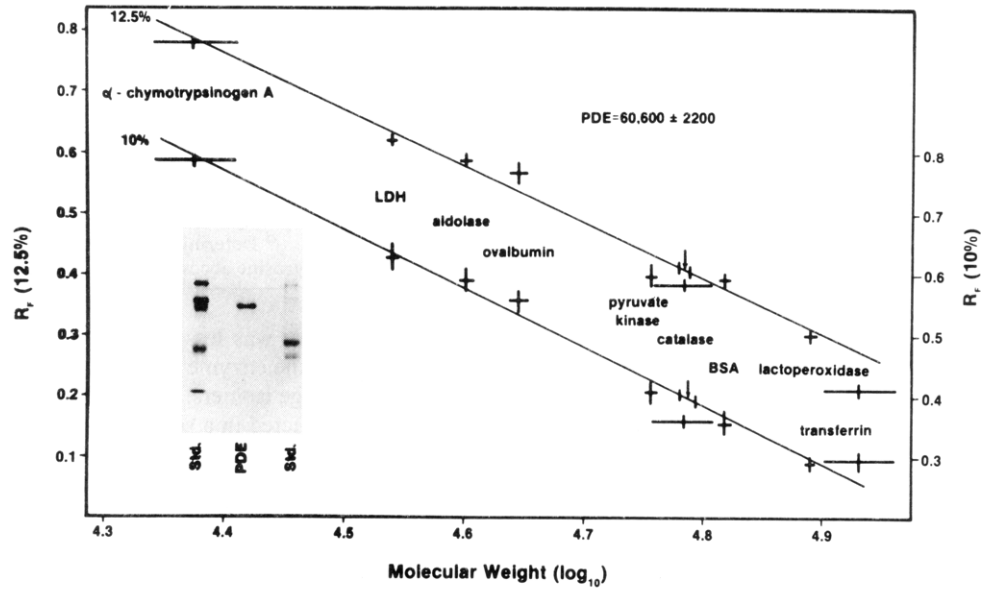


FIGURE 5: $NaDodSO_4$–acrylamide slab gel electrophoresis of purified cAMP phosphodiesterase. Electrophoresis of 10 μg of the enzyme protein and 10 μg of the nine indicated standard proteins, staining, and destaining were as described under Experimental Procedures. The $NaDodSO_4$-determined molecular weight was calculated by linear regression analysis of both the 10 and 12.5% gel patterns.

denaturation for gel electrophoresis.

The overall recovery for activity measured by using 0.25 μM cAMP as substrate is ~4%, and ~20 mg of enzyme protein can be obtained from 20 dog kidneys. The increase in specific activity is 125-fold from the original homogenate. Even though this value is rather low, we believe that the evidence presented below makes it very unlikely that cAMP phosphodiesterase has copurified with another protein(s) and suggest that the purified protein is enzyme protein, albeit not necessarily in a fully active state.

*Criteria of Purity and Properties of the Purified Enzyme.* $NaDodSO_4$–acrylamide slab gel electrophoresis of enzyme purified through the Sephacryl step (Figure 5) shows a single protein band when stained with Coomassie Blue or Stains All. Densitometric scans of the stained gels showed no other detectable staining. When compared to nine standard proteins using 10 or 12.5% gels, the apparent molecular weight of the enzyme is 60 600 ± 2200. The molecular weight was similar with 7.5% gels, suggesting that the protein had free mobility under these conditions. Similar results were obtained when PMSF and TLCK were included during boiling in $NaDodSO_4$ or when the $Cl_3AcOH$-precipitated protein was resuspended in $NaDodSO_4$ without boiling and applied directly to the stacking gel.

High-speed sedimentation equilibrium centrifugation of the enzyme preparation using 100 mM $K_2PO_4$ buffer in 30%

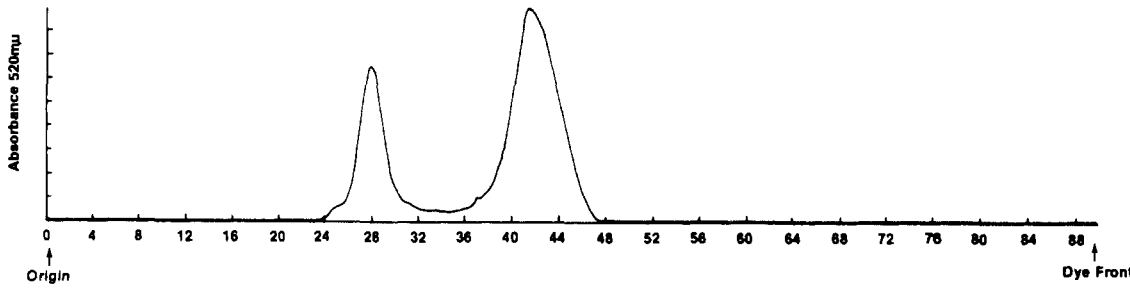


FIGURE 6: 10% acrylamide gel electrophoresis of purified cAMP phosphodiesterase. Purified enzyme (20 μg) was dialyzed and electrophoresed on 10% acrylamide slab gels in 30% ethylene glycol as indicated under Experimental Procedures. The densitometer tracing shown is integrated to the highest peak.

ethylene glycol showed a linear ln $C$ vs. $r^2$ plot. The weight average molecular weight calculated using a partial specific volume of 0.723 was 48 000.

Sedimentation velocity experiments conducted using EG buffer gave a single symmetrical peak. Purified enzyme (3 mg/mL) in EG buffer was centrifuged at 60000$g$ at 20 °C with a Beckman Model E using a double sector cell with EG buffer as the reference. Because of the curvature of the base line produced by this buffer system, the sedimentation coefficient was determined by comparison with BSA in the same buffer. A value of 3.6 S was obtained by this procedure (data not shown).

The sedimentation coefficient was also determined by using 5–20% linear sucrose gradients. Because its density is higher than that of 5% sucrose, enzyme in EG buffer was diluted 1:40 in 50 mM $K_2PO_4$ (pH 6.8) in order to layer 200 μL on the gradients. The standard proteins (2 mg) in phosphate–EG buffer were run in pairs in three adjacent gradients. Centrifugation was at 49 000 rpm for 14 h in a Beckman SW 50.1 rotor. The protein and activity profiles obtained were coincident, indicating homogeneity of the enzyme. When compared by linear regression analysis to six standard proteins, cAMP phosphodiesterase had an $s_{20,w}$ of 3.2–3.4 S. However, its activity was unstable and total recovery was less than 10%. The diffusion coefficient was $8.6 \times 10^{-7}$ cm$^2$/s, as determined by plotting the distance of migration vs. the log of the diffusion coefficient of the same standards.

Sephacryl S-200 (SF) gel filtration was used to determine the diffusion coefficient of cAMP phosphodiesterase under the same conditions as those used to determine the sedimentation coefficient in order to calculate the molecular weight by using the equation $M_r = RTs/D(1 - \bar{v}\rho)$. By use of EG buffer containing 0.5 M sodium acetate, a plot of $K_{av}$ vs. log $1/D_{20,w}$ of six standard proteins gave a diffusion coefficient of $6.1 \times 10^{-7}$ cm$^2$/s. The activity and protein profiles were again coincident, the activity recovery was 100%, and the calculated molecular weight was 53 000. The Stokes' radius was calculated from $a = kT/6\pi\eta D$ as $35 \times 10^{-8}$ Å, and the $f/f_0$ was 1.406 from $f/f_0 = a/(3\bar{v}M_r/4\pi N)^{1/3}$.

Calculation of the molecular weight by using the diffusion coefficient determined by linear sucrose gradients ($8.6 \times 10^{-7}$ cm$^2$/s) gave a value of 38 045. The Stokes' radius was $25 \times 10^{-8}$ Å and the frictional coefficient 1.12 under these conditions.

Native acrylamide gel electrophoresis has proved difficult because dialysis against aqueous buffers or dilution, steps necessary to reduce the ionic strength of the protein sample for electrophoretic mobility, results in unstable activity. The activity was partially stabilized by dialysis against ethylene glycol and use of 10% acrylamide slab gels polymerized in 30% ethylene glycol. Figure 6 shows the densitometric scan of Coomassie Blue stained gels. Each band showed activity, but the total recovery was less than 10%. Unlike $NaDodSO_4$ electrophoresis, the enzyme shows two bands, suggesting the presence of charge isomers. Visualization of the bands with Stains All, conducted in a separate experiment, showed that the faster migrating band stained a more intense blue than did the trailing band, suggesting that one portion of the enzyme population may be more acidic than the other.

Table II: Amino Acid Composition of High-Affinity cAMP Phosphodiesterase

| amino acid | mol % residue | residues/mol of PDE[a] |
|---|---|---|
| Asp | 15.9 | 72 |
| Thr | 3.5[b] | 18 |
| Ser | 8.5[b] | 49 |
| Glu | 13.8 | 57 |
| Gly | 6.8 | 55 |
| Ala | 5.4 | 34 |
| Cys | 0 | 2[d] |
| Val | 7.9[c] | 41 |
| Met | 0.8 | 3 |
| Ile | 4.8[c] | 22 |
| Leu | 8.8[c] | 41 |
| Tyr | 3.5 | 12 |
| Phe | 4.4 | 16 |
| Trp | 0 | 16[e] |
| Lys | 15[b] | 62 |
| His | 2.5 | 10 |
| Arg | 3.5[b] | 12 |
| Pro | 4.9 | 26 |
| $NH_3$ | 18.2 | |

[a] Calculated as nearest whole integer by assuming 60 600 g/mol of phosphodiesterase (PDE). [b] Extrapolated to zero time by using 24-, 48-, and 72-h hydrolysis times. [c] Complete hydrolysis assumed after 72 h. [d] Determined by DTNB titration. [e] Determined as ratio to tyrosine according to Bencze & Schmid (1957).

The amino acid composition of the enzyme is shown in Table II. The mole percent residue values integrated well to residues per mole by assuming the $NaDodSO_4$-determined molecular weight. Hydrolyses of 24, 48, and 72 h were necessary because of the lability of threonine, serine, lysine, and arginine and the resistance to hydrolysis of the hydrophobic amino acids, which required extrapolations to zero time. The most prominent finding was the high content of aspartic and glutamic acids. This apparent acidity of the enzyme was consistent with the electrophoretic staining behavior. The p$I$ of the protein was subsequently determined to be 4.8 ± 0.1 by isoelectric focusing (Figure 7).

Kinetic studies of the purified enzyme showed a linear double-reciprocal plot for cAMP hydrolysis by using 0.2–100 μM substrate concentrations. The purified cAMP phosphodiesterase showed a high affinity ($K_m$ = 2.2 μM) for cAMP. The maximum velocity of the purified enzyme is 95 nmol/(min mg) under these conditions of activity analysis. The $K_m$ for cGMP hydrolysis was 312 μM, and cGMP was competitive

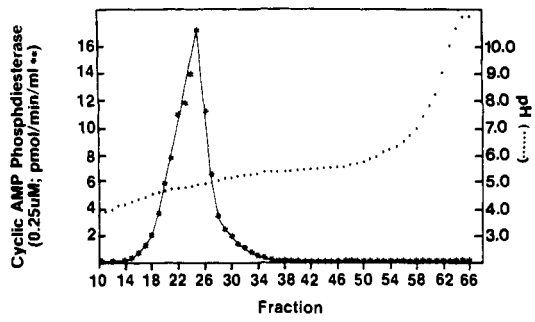


FIGURE 7: Isoelectric focusing of purified cAMP phosphodiesterase. Enzyme (200 μg) was layered upon a 10–40% glycerol gradient in an LKB 8101 column. The gradient contained 30% ethylene glycol, 1 mM DTT, 0.5% Brij 35, and ampholines of 1.4% pH 3.5–5.0 and 1.4% pH 3.5–10.0 with 37 mM Hepes and 37 mM Bicine. Focusing was for 65 h as indicated under Experimental Procedures.

Table III: Physical and Kinetic Properties of High-Affinity cAMP Phosphodiesterase

| property | value | method |
|---|---|---|
| $K_m$ (cAMP) (μM) | 2.2 | Lineweaver-Burk |
| max velocity [nmol/ (min mg)] | 95 | Lineweaver-Burk |
| | | no CDR sensitivity |
| absorbance (1%/280 nm] | 9.4; 22 | spectrophotometry (0.1 N NaOH; 40 mM Tris-HCl, pH 8.0) |
| sedimentation coeff ($s_{20,w}$, $\times 10^{-13}$ s) | 3.6 | sedimentation velocity |
| diffusion coeff ($D_{20,w}$, $\times 10^{-7}$ cm$^2$ s) | 6.14 | gel filtration |
| partial sp vol (20 °C) | 0.732 | amino acid analysis |
| mol wt | 60 600 | $NaDodSO_4$ gel electrophoresis |
| | 48 000 | sedimentation equilibrium |
| | 53 300 | calcd using $s$ and $D$ |
| Stokes' radius ($\times 10^{-8}$ Å) | 35 | |
| $f/f_0$ | 1.406 | |
| isoelectric point | 4.8 | isoelectric focusing |

with cAMP hydrolysis ($K_i \simeq 500$ μM). More detailed studies of the kinetic properties of the purified enzyme will be presented elsewhere.

The purified high-affinity enzyme was unaffected by CDR prepared according to Lin et al. (1974) or purified CDR obtained from Dr. D. M. Watterson. Additional studies in collaboration with Dr. C. B. Klee (Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD) revealed no sensitivity of the purified enzyme to purified CDR under conditions where low-affinity, brain cAMP–cGMP phosphodiesterase was activatable. Dr. Klee has determined that purified inhibitory protein also has no effect on the activity of this enzyme and that when coelectrophoresed on 7–15% $NaDodSO_4$–acrylamide gels, the purified enzyme migrates as a single band slightly faster than the 61 000 molecular weight subunit of the inhibitory protein (Klee & Krinks, 1978). Moreover, the purified high-affinity enzyme had no effect on the inhibitory action of the inhibitory protein on CDR-sensitive brain cAMP phosphodiesterase. A general summary of the properties of purified dog kidney, high-affinity cAMP phosphodiesterase is shown in Table III.

## Discussion

The existence of multiple forms of cAMP phosphodiesterase was proposed as one explanation for the anomalous kinetics observed for hydrolysis of cAMP by tissue homogenates (Thompson & Appleman, 1971a,b). This hypothesis has since been supported by a variety of physical separation techniques applied to many mammalian tissues, but in recent years it has also become apparent that conditions of tissue homogenization and the subsequent treatment of subcellular fractions can dramatically influence the number and properties of forms isolated, inclusive to the problems associated with the technique employed. Therefore, purification of high- and low-affinity cAMP or cGMP phosphodiesterase forms, which has not been previously achieved, has become paramount to understand the biochemical nature of this complex enzyme system.

The purificaton strategy developed in these studies was to separate the high-affinity cAMP specific enzyme from the bulk of the lower affinity cAMP–cGMP enzyme form. In addition, a buffer system was developed to stabilize enzyme activity upon isolation, a severe drawback in many previous studies [e.g., Huang & Kemp (1971) and Ho et al. (1977)]. The high-affinity enzyme form was then purified by conventional techniques. Subsequently, purification of the low-affinity form of the enzyme will provide a complete definition of the biochemical parameters of the holoenzyme system. The high-affinity enzyme form is of additional physiological and pharmacological interest because it is thought to be the hormone-sensitive enzyme form, its substrate affinity would dictate primary participation in biological regulatory processes, and high-affinity activity predominates in the plasma membrane of many cell types (Thompson & Strada, 1978).

Inclusion of 30% ethylene glycol in the complex buffer system used for chromatography and enzyme storage appears to be the main factor in stabilization of either of the forms of the enzyme. The Stokes' radius of the purified protein is much smaller ($24 \times 10^{-8}$ vs. $35 \times 10^{-8}$ Å) and the frictional coefficient closer to 1 (1.12 vs. 1.41) when calculated with a diffusion coefficient determined with 1% ethylene glycol as compared to 30% ethylene glycol in the buffer. Since the native conformations of many proteins are thought to be stable to concentrations of ethylene glycol in excess of 75% (Tanford, 1968), one explanation of the stabilizing effect is that it results from a more ellipsoid shape of cAMP phosphodiesterase due to the lower dielectric constant of the medium and diminished solvent-charge interactions. Other polyhydric compounds such as sucrose or glycerol did not provide enzyme stability equivalent to that with ethylene glycol. This compound has the additional advantage that it did not present a viscosity problem at higher concentrations and lower temperatures, and it also provided a storage media for the enzyme that does not freeze at −20 °C. Freezing and thawing in an aqueous medium is detrimental to activity.

The presence of the protease inhibitor, TLCK, throughout purification is critical in minimizing the appearance of active and inactive protein fragments. Terai et al. (1976) have also reported that partial purification of a high-affinity cAMP phosphodiesterase from rat pancreas requires protease inhibitors. Several studies have indicated that proteolysis is an important factor in enzyme form analysis (Patterson et al., 1976; Sakai et al., 1978; Moss et al., 1978; Terai et al., 1976; Epstein et al., 1978). Limited proteolysis of higher molecular weight enzyme forms results in a low molecular weight enzyme with properties similar to the purified enzyme (Epstein et al., 1978).

Many of the properties of the high-affinity enzyme revealed in less pure preparations have been preserved through the purification procedure described here. These properties, most of which are distinct from those of low-affinity cAMP or cGMP phosphodiesterase, include (1) high affinity for cAMP as substrate (2.2 μM), (2) relative specificity for cAMP vs. cGMP as substrate (140:1), (3) competitive inhibition by cGMP of cAMP hydrolysis ($K_i$ = 530 μM), (4) lack of activation by lower concentrations of cGMP or by proteolytic

enzymes, (5) lack of effects of CDR or inhibitor proteins on activity, and (6) a molecular weight lower than that of the low-affinity system. The negative cooperativity observed for cAMP hydrolysis with cruder enzyme preparations was not apparent when the purified enzyme was assayed under these conditions. It remains to be established if some regulatory component may have been removed during purification of the enzyme, if different assay conditions are required to observe cooperative kinetics, or if the apparent cooperativity is due to contamination with low-affinity enzyme which results in such kinetic behavior (Thompson & Appleman, 1971b). The high-affinity enzyme partially purified from skeletal muscle also showed linear kinetics for cAMP hydrolysis (Huang & Kemp, 1971).

The high-affinity enzyme form appears to be homogeneous according to the criteria of $NaDodSO_4$–acrylamide gel electrophoresis, sedimentation equilibrium, gel filtration, and sedimentation velocity. However, the molecular activity of the enzyme is 5757 [calculated using $V_{max}$ = 95 nmol/(min mg) and a molecular weight of 60 600] and the relative increase in specific activity is only 125-fold from homogenate to apparent homogeneity. Since the specific activity remains constant with several additional purification procedures, a significant percentage of the purified enzyme may be inactive. Alternatively, an impurity may have copurified with the enzyme. Native gel electrophoresis did show two bands of protein, but since activity was apparent in each band and was distributed in a similar percentage, this alternative is not very probable. No evidence was obtained that the enzyme is bound to another protein. In addition, a substantial portion of the hydrolysis of cAMP by the homogenate is due to the low-affinity enzyme form, which artifically lowers the fold purification.

Determinations of the molecular weight of the high-affinity enzyme showed a variance from 48 000 to 60 600. No evidence for active or inactive, higher or lower molecular weight species was obtained, indicating that this protein is the catalytic portion of the enzyme. The $NaDodSO_4$–acrylamide gel electrophoresis molecular weight of 60 600 could be higher than the 48 000 obtained from sedimentation equilibrium if the high negative charge of this acidic protein precluded complete $NaDodSO_4$ binding. On the other hand, the sedimentation equilibrium value might be low if the medium employed had some anomalous effect on the partial specific volume of the protein. The calculated molecular weight value of 53 000 using sedimentation and diffusion coefficients obtained by sedimentation velocity and gel filtration could have been influenced as well by slight inaccuracies in the determination of these values by comparative methods. Although the data were not shown, direct estimation of the molecular weight by gel filtration using a comparison with six standard proteins was 64 500, suggesting that the asymmetry of the protein in EG buffer should also be considered. Although it is not clear whether a high-affinity enzyme form similar to the dog kidney form is equally apparent in all mammalian tissues, previous estimates primarily by gel filtration of the size of a kinetically similar form range from 70 000 to 180 000 (Bergstrand et al., 1978; Loten et al., 1978; Kakiuchi et al., 1975; Terai et al., 1976; Patterson et al., 1976; Hidaka & Asano, 1976). The molecular weight of the purified enzyme is remarkably similar to 70 000 estimated by Jard & Bernard (1970) and Kakiuchi et al. (1975) by gel filtration of crude rat kidney supernatants.

Partial purification of one form of the low-affinity enzyme, a calcium-dependent form, has been achieved by others, and the molecular weight of the complex is variably reported from several laboratories to be 130 000–250 000. Klee & Krinks (1978) have reported in preliminary experiments that the low-affinity enzyme from brain, obtained by selective binding to CDR affinity columns, consists of 61 000, 59 000, and 15 000 molecular weight subunits by $NaDodSO_4$–acrylamide gel electrophoresis. Somewhat similar findings were previously observed by Watterson & Vanaman (1976). The molecular weight obtained by $NaDodSO_4$–acrylamide gel analysis in our studies for the high-affinity enzyme, 60 600, suggests that the low-affinity system may be a complex of one or more high-affinity subunits which become modified upon association with subunits of the inhibitor protein (61 000 and 15 000), CDR (18 000), and/or other factors to provide the observed kinetic behavior and effector sensitivities. Our previous studies on human peripheral blood lymphocytes which contain only the high-affinity form of the enzyme led us to a similar hypothesis (Thompson et al., 1976), and the heterogeneity of kinetically similar human lung enzymes upon gel filtration led Bergstrand et al. (1978) to a similar postulate. Further studies are needed to analyze these potentially fortuitous results, but this working hypothesis is based upon defined properties of the purified high-affinity enzyme as the basic catalytic subunit of the system should prove useful in helping to understand the biochemistry and eventually the physiological regulation of cyclic nucleotide phosphodiesterases in mammalian tissues.

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## References

Appleman, M. M., & Terasaki, W. L. (1975 *Adv. Cyclic Nucleotide Res. 5*, 153.

Bencze, W. L., & Schmid, K. (1957) *Anal. Chem. 29*, 1193.

Bergstrand, H., Lundquist, B., & Schurmann, A. (1978) *J. Biol. Chem. 253*, 1881.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Brostrom, C. O., & Wolff, D. J. (1976) *Arch. Biochem. Biophys. 172*, 301.

Butcher, R. W., & Sutherland, E. W. (1962) *J. Biol. Chem. 237*, 1244.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, p 370, Reinhold, New York.

Davis, C. W., & Kuo, J. F. (1977) *J. Biol. Chem. 252*, 4078.

Dousa, T., & Rychlik, I. (1970) *Biochim. Biophys. Acta 204*, 10.

Ellman, G. J. (1959) *Arch. Biochem. Biophys. 82*, 70.

Epstein, P. M., Mills, J. S., Ross, C. P., & Strada, S. J. (1977) *Cancer Res. 37*, 4016.

Epstein, P. M., Pledger, W. J., Gardner, E. A., Stancel, G. M., Thompson, W. J., & Strada, S. J. (1978) *Biochim. Biophys. Acta 527*, 442.

Filburn, C. R., & Sacktor, B. (1976) *Arch. Biochem. Biophys. 174*, 249.

Fujimoto, M., Ichikawa, A., & Tomita, K. (1974) *Arch. Biochem. Biophys. 161*, 54.

Goren, E. N., & Rosen, O. M. (1972) *Arch. Biochem. Biophys. 153*, 384.

Green, M. W., Pastewks, J. V., & Peacock, A. C. (1973) *Anal. Biochem. 56*, 43.
Gulyassy, P. F., Ferrand, J. R., & Gugler, H. D. (1975) *Kidney Int. 8*, 284.
Hegenaver, J., Ripley, L., & Nace, G. (1977) *Anal. Biochem. 78*, 308.
Hidaka, H., & Asano, T. (1976) *Biochim. Biophys. Acta 429*, 485.
Ho, H. C., Wirch, E., Stevens, F. C., & Wang, J. H. (1977) *J. Biol. Chem. 252*, 43.
Huang, Y. C., & Kemp, R. G. (1971) *Biochemistry 10*, 2278.
Jard, S., & Bernard, M. (1970) *Biochem. Biophys. Res. Commun. 41*, 781.
Jarrett, H. W., & Penninston, J. T. (1978) *J. Biol. Chem. 253*, 4676.
Kakiuchi, S., Yamazaki, R., Teschima, T., Uenishi, K., & Miyamoto, E. (1975) *Biochem. J. 146*, 109.
Klee, C. B., & Krinks, M. H. (1978) *Biochemistry 17*, 120.
Laemmli, V. K. (1970) *Nature (London) 227*, 680.
Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974) *J. Biol. Chem. 249*, 4943.
Loten, E. G., Assimacopovlos-Jeannet, F. D., Exton, J. H., & Park, C. R. (1978) *J. Biol. Chem. 253*, 746.
Mazin, A. L., Sulimova, G. E., & Vanyushin, B. F. (1974) *Anal. Biochem. 61*, 62.
Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320.
Miyake, M., Daly, J. W., & Creveling, C. R. (1977) *Arch. Biochem. Biophys. 181*, 39.
Morishima, I. (1974) *Biochim. Biophys. Acta 370*, 227.
Moss, J., Manganiello, V. C., & Vaughan, M. (1978) *Biochim. Biophys. Acta 541*, 279.
Patterson, W. D., Hardman, J. G., & Sutherland, E. W. (1976) *Mol. Cell. Endocrinol. 5*, 51.
Rosen, O. M. (1970) *Arch. Biochem. Biophys. 137*, 435.
Sakai, T., Makino, H., & Tanaka, R. (1978) *Biochim. Biophys. Acta 522*, 477.
Schacterle, G. R., & Pollack, R. L. (1973) *Anal. Biochem. 51*, 654.
Schubart, U. K., Udem, L., Baum, S. G., & Rosen, O. M. (1974) *Mol. Cell. Endocrinol. 1*, 227.
Sharma, R. K., Desai, R., Thompson, T. R., & Wang, J. H. (1978) *Can. J. Biochem. 56*, 598.
Siegel, F. J., & Egrie, J. C. (1977) *Biochim. Biophys. Acta 483*, 348.
Strada, S. J., & Thompson, W. J. (1978) *Adv. Cyclic Nucleotide Res. 9*, 265.
Tanford, C. P. (1968) *Adv. Protein Chem. 23*, 209.
Terai, M., Furihata, C., Matsushima, T., & Sugimura, T. (1976) *Arch. Biochem. Biophys. 176*, 621.
Thompson, W. J., & Appleman, M. M. (1971a) *J. Biol. Chem. 246*, 3145.
Thompson, W. J., & Appleman, M. M. (1971b) *Biochemistry 10*, 311.
Thompson, W. J., & Strada, S. J. (1978) *Recept. Horm. Action 3*, 553.
Thompson, W. J., Ross, C. R., Pledger, W. J., Strada, S. J., Banner, R. L., & Hersh, E. M. (1976) *J. Biol. Chem. 251*, 4922.
Thompson, W. J., Terasaki, W. L., Epstein, P. M., & Strada, S. J. (1979) *Adv. Cyclic Nucleotide Res. 10*, 69.
Udenfriend, S. (1969) in *Fluorescence Assay in Biology and Medicine II*, p 248, Academic Press, New York.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgrnber, W., & Weigele, M. (1972) *Science 178*, 871.
Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., Kutzner, R., Delforge, A., & Gristophe, H. (1977) *Eur. J. Biochem. 81*, 379.
Van Inwegen, R. G., Swafford, R. L., Strada, S. J., & Thompson, W. J. (1977) *Arch. Biochem. Biophys. 178*, 58.
Wang, J. H., & Desai, R. (1977) *J. Biol. Chem. 252*, 4175.
Watterson, D. M., & Vanaman, T. C. (1976) *Biochem. Biophys. Res. Commun. 73*, 40.
Watterson, D. M., Harrelson, W. G., Keller, P. M., Shariff, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501.
Wells, J. N., & Hardman, J. G. (1977) *Adv. Cyclic Nucleotide Res. 8*, 119.