Subunit Structure and Catalytic Properties of Bovine Brain Ca^{2+}-Dependent Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: Ca^{2+}-dependent cAMP phosphodiesterase has been purified 3000-fold to apparent electrophoretic and chromatographic homogeneity by affinity chromatography on Ca^{2+}-dependent regulatory protein coupled to Sepharose followed by gel filtration on Sephadex G-200 and chromatography on hydroxylapatite. The purified enzyme contains three subunits of molecular weights of 61 000, 59 000, and 15 000. The 59 000 molecular weight polypeptide alone is associated with catalytic activity toward cAMP and cGMP as well as Ca^{2+} stimulation. The other two subunits appear to be those of the inhibitory protein previously described in this laboratory which seems to physically interact with the enzyme. This purified enzyme displays the catalytic properties characteristic of the "high K_m" enzyme; it obeys Michaelis–Menten kinetics in the presence of Ca^{2+} and CDR. The Ca^{2+}-dependent regulatory protein is shown to increase the V_m value by eight- to tenfold and to decrease the K_m value two- to threefold under optimal conditions. The enzyme is also stimulated by NH_4Cl which increases the K_m and the V_m of the enzyme for cAMP both in the presence and absence of Ca^{2+}-dependent regulator. Imidazole, another activator of the enzyme at high cAMP concentrations, increases V_m and K_m for cAMP and inhibits the interaction of the enzyme with the Ca^{2+}-dependent regulatory protein and can thereby become an inhibitor at 10^{-6} M cAMP and nonsaturating concentrations of CDR.

The cAMP phosphodiesterase from mammalian tissues has been studied extensively in many laboratories (for a review of the literature, see Wells & Hardman, 1977), but a definitive analysis of the mechanism of the reaction catalyzed by this enzyme and of its regulation must await the availability of a homogeneous enzyme preparation. As pointed out by Thompson & Appleman (1971), the difficulties encountered in the purification of this enzyme may result in part from the existence of multiple molecular forms. One of these forms is specifically activated by an endogenous regulatory protein (Cheung, 1970, 1971; Kakiuchi et al., 1975; in a Ca^{2+}-dependent process (Kakiuchi et al., 1975; Teo & Wang, 1973). The regulatory protein (Ca^{2+}-dependent regulator, or CDR)[1] interacts with Ca^{2+}, and the Ca^{2+}–CDR complex activates the enzyme (Kakiuchi et al., 1973; Teo & Wang, 1973; Lin et al., 1975; Wolff & Brostrom, 1974); such an activated enzyme will be referred to as Ca^{2+}-dependent, or CDR-dependent, cyclic nucleotide phosphodiesterase. The enzyme from heart has been purified by Ho et al. (1977) on the basis of its ability to form a reversible Ca^{2+}-dependent complex with CDR. Various attempts to purify the enzyme from brain, using affinity chromatography on CDR coupled to Sepharose, have yielded only partially purified enzymes (Watterson & Vanaman, 1976; Miyake et al., 1977). The copurification of the phosphodiesterase with other proteins on CDR–Sepharose (Klee, 1977a; Klee & Krinks, 1978)[2] is not surprising in view of the recent reports of the ability of CDR to regulate a number of different enzymes (Brostrom et al., 1975; Cheung et al., 1975; MacIntyre & Green, 1977; Berridge, 1975; Vanaman et al., 1976; Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977; Dedman et al., 1977; Dabrowska et al., 1978; Yagi et al., 1978; Waisman et al., 1978). These multiple roles of the Ca^{2+}-dependent regulator make it even more important to understand its mode of action in the phosphodiesterase activation since this activation mechanism may be shared by other enzymatic systems. As a first step in our attempt to elucidate the mechanism of action of CDR, we describe in this paper the purification of Ca^{2+}-dependent phosphodiesterase of bovine brain to apparent electrophoretic and chromatographic homogeneity as well as the characterization of its subunit structure and some of its kinetic properties.

Materials and Methods

Bovine brain Ca^{2+}-dependent regulator was prepared as previously described (Klee, 1977b). Cyclic guanosine 3′,5′-monophosphate (cGMP) was a product of Calbiochem. Guanosine 5′-monophosphate (GMP) and [8-^{14}C]GMP were obtained from Schwarz/Mann; [8-^{3}H]cGMP and [8-^{3}H]-cAMP from ICN Pharmaceuticals were used without prior purification (the blank values in the assays were at most 1%, and more than 90% of the radioactivity coeluted with cAMP upon chromatography on the AG-50W-X8 columns). All other chemicals were as previously described (Klee, 1977b).

Enzyme Assays. The standard assay procedure was as previously described (Klee, 1977b). One enzyme unit catalyzes the formation of 1 μmol of 5′-AMP per min at 30 °C in an incubation mixture (0.1 mL) containing 40 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 0.05 mM $CaCl_2$, 0.1 mM dithioerythritol, 2×10^{-4} M [^{3}H]cAMP (specific activity 2000–3000 cpm/nmol), 10^{-7} M [^{14}C]AMP (100 000 cpm/nmol), 10^{-6} M CDR, and 0.1 mg of bovine serum albumin per mL. Addition of bovine serum albumin was required to obtain an optimum level of Ca^{2+}-dependent activation. Basal activity measured in the presence of 0.025 mM EGTA was not affected by bovine serum albumin. For kinetic measurements, incubation times (3–40 min) were such that less than 20% of the substrate was utilized; addition of NH_4Cl (0.2 M) to the incubation mixture caused a negligible (minus 0.02) change in pH; imidazole

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[1] Abbreviations used: CDR, Ca^{2+}-dependent regulator protein of adenosine 3′,5′-monophosphate phosphodiesterase, also called activator protein and modulator protein; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; mol wt, molecular weight.

[2] Bovine brain, human platelet, and turkey gizzard myosin light-chain kinases were also purified by affinity chromatography on CDR coupled to Sepharose (D. Hathaway, C. Klee, & R. Adelstein, unpublished results).

solutions were adjusted to pH 8.0 prior to addition to the assay. Enzyme was diluted prior to assay in 0.04 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM dithioerythritol, and 50% (v/v) glycerol without significant loss of activity. For each point the substrate concentrations were corrected for the amount of product formed. The kinetic constants for cAMP were obtained by fitting the data to the relationship[3] $v = VA/(K + A)$. An interactive curve fitting program, MLAB, was used with a PDP-10 digital computer (Knott & Reese, 1971).

cGMP phosphodiesterase was assayed by a modification of the cAMP phosphodiesterase assay. After application of the sample, AG-50W-X8 columns were washed twice with 2 mL and twice with 1 mL of 0.015 M HCl, 0.025 M KCl (pH 2.0); thereafter 5′-GMP was eluted in a 60–80% yield with 4 mL of H_2O.

Protein concentrations were measured spectrophotometrically by absorption at 280 nm. (One A_{280} unit is the amount of protein in 1 mL of solution having an A_{280nm} of 1.0 using a path length of 1 cm.) For the purified enzyme (fraction VII), 1 A_{280} was shown to correspond to 1.06 ± 0.1 mg of protein by amino acid analysis.

Gel Electrophoresis. Disc gel electrophoresis under nondenaturing conditions was performed according to Davis (1964). After electrophoresis the gel was sliced in 1.5-mm sections and activity was measured by incubating each slice in the standard incubation mixture. Alternatively, the enzyme or proteins could be eluted by soaking each slice in 0.05 mL of 0.04 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, 0.1 mM dithioerythritol, 1 mM $MgCl_2$, 10% (v/v) glycerol, and bovine serum albumin (40 μg/mL) overnight at 0–4 °C. The recovery of enzyme activity in both cases varied between 50 and 70%. Omission of carrier protein from the eluting buffer resulted in a decrease of the enzyme recovery, particularly in fractions with very low protein concentration. Gel electrophoresis in the presence of $NaDodSO_4$ was done as described previously, using a 5–15% gradient of acrylamide (Klee & Krinks, 1978).

Results

Purification Procedure

Crude CDR-dependent cAMP phosphodiesterase was prepared by a modification of the procedure of Cheung & Lin (1974) as previously described (Klee & Krinks, 1978). All buffer solutions contained 1.5 mL of a solution of phenylmethanesulfonyl fluoride (50 mg/mL in dimethyl sulfoxide) per L, up to and including the DEAE-cellulose step. Bovine brains were obtained from the slaughterhouse and were used as such or stored frozen at −70 °C. Frozen brains were thawed overnight at 0–4 °C. Ammonium sulfate fractionation was done in the presence of 1 mM EDTA. The 30–60% ammonium sulfate fraction was resuspended in a minimum volume of 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM imidazole and 1 mM $MgCl_2$ and was dialyzed overnight against 10 volumes of the same buffer. The dialysis fluid was changed once. The conductivity of the dialyzed sample (fraction II) was 3.2 mS measured at 0 °C.

DEAE-Cellulose Chromatography. This step was carried out as previously described (Klee & Krinks, 1978). Fractions of 500 mL were collected during the enzyme elution. The enzyme was eluted in a 75% yield in the 0.15 and 0.3 M $(NH_4)_2SO_4$ buffer washes. Only the 0.15 M $(NH_4)_2SO_4$ fraction was used for the subsequent steps. The enzyme was concentrated by ammonium sulfate precipitation (436 g/L) in the presence of 1 mM EDTA. The pellet was resuspended in a minimum volume of 0.04 M Tris-HCl buffer (pH 8) containing 3 mM $MgCl_2$, 0.05 M NaCl, and 0.1 mM dithioerythritol and was dialyzed against 10 volumes of the same buffer. The dialysis fluid was changed once. At this stage, the enzyme (fraction III) could be stored frozen at −70 °C for 5 days without loss of activity.

Affinity Chromatography on CDR Coupled to Sepharose. CDR–Sepharose was prepared as previously described (Klee & Krinks, 1978), except that 9.5 mg of protein in 2 mL of 0.1 M borate buffer (pH 8.2) was used for 12 mL of activated Sepharose. The dialyzed DEAE-cellulose fraction III (from 500 g of brain) was made 0.2 mM in $CaCl_2$ and was loaded onto a column of CDR–Sepharose (1.6 × 6 cm) equilibrated with 0.04 M Tris-HCl buffer (pH 8) containing 0.05 M NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.1 mM dithioerythritol at a flow rate of 30 mL/h. The column was then washed with 35 mL of equilibrating buffer and 35 mL of the same buffer made 0.2 M in NaCl. The enzyme was eluted with 45 mL of 0.04 M Tris-HCl buffer (pH 8) containing 0.2 M NaCl, 1 mM $MgCl_2$, 2 mM EGTA, and 0.1 mM dithioerythritol. The fraction size was 10 mL prior to the last two washes and 2 mL thereafter. At this step the enzyme was routinely collected in polyethylene tubes. A small proportion of the enzyme (12%) did not bind to the column. The bulk of the units recovered from the column was eluted as a sharp peak with the EGTA-containing buffer in a 40% yield. Fractions containing 1 or more A_{280} units/mL were pooled (fraction IV) and immediately subjected to the next step.

Gel Filtration on Sephadex G-200 Superfine. Fraction IV was applied to a column of Sephadex G-200 superfine (1.6 × 80 cm) equilibrated with 0.04 M Tris-HCl (pH 8) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM dithioerythritol, and 20% (v/v) glycerol (buffer A). The flow rate was 2 mL/h. The elution profile of the protein is shown in Figure 1. Most of the enzyme is eluted after the bulk of the protein monitored by absorption at 280 nm. A shoulder on the ascending part of the enzyme peak was reproducibly observed, although it was quantitatively variable. The material applied to the column as well as various fractions eluted from the column were subjected to gel electrophoresis under denaturing conditions. The electrophoretic pattern is shown at the top of Figure 1. Most of the polypeptides adsorbed on the CDR–Sepharose column (fraction IV) did not appear to be associated with enzyme activity since they were partially resolved from it by the gel filtration step. Large polypeptides (235 000 and 150 000 mol wt) were eluted before the enzyme (tubes 42–46). The two polypeptides with molecular weights 61 000 and 15 000 eluted last (tubes 55–60) are the subunits of the inhibitory protein described previously (Klee & Krinks, 1978). One protein band which has an apparent molecular weight of 59 000 appears to correlate with enzyme activity (fraction 46, 52, and 55). Fractions with the highest specific activities were pooled as shown in the figure (fraction V). They contained only 60% of the enzyme recovered from the column.

Hydroxylapatite Chromatography. In view of the instability of fraction V (a 75% loss of activity was observed in 5 days at 0–4 °C; the enzyme was also unstable at −70 °C in the presence of 50% glycerol), the next purification step was carried out immediately. Fraction V was loaded onto a 0.9 × 2 cm column of hydroxylapatite equilibrated in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, 0.1 mM dithioerythritol and 20% (v/v) glycerol at a flow rate of 30 mL/h. The enzyme was washed onto the column with 5 mL

[3] The nomenclature is that of Cleland (1963).

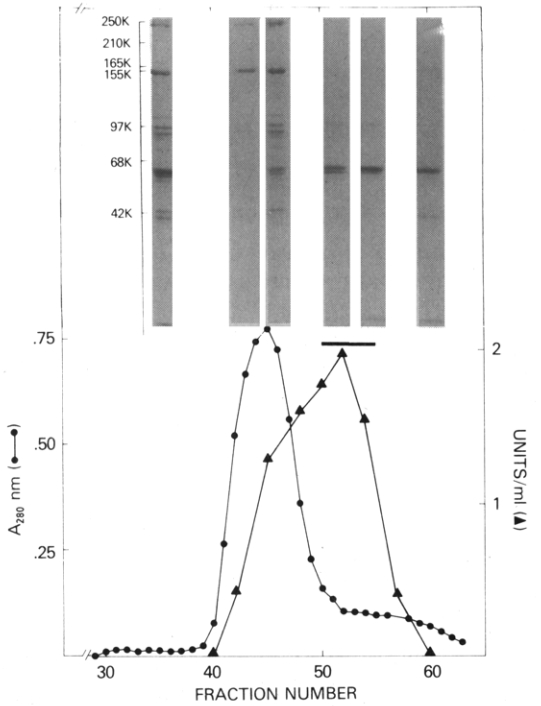

FIGURE 1: Gel filtration of cAMP phosphodiesterase. Bottom: Fraction IV was applied to a 1.6 × 80 cm column of Sephadex G-200 superfine, as described in Materials and Methods. The fraction size was 1.7 mL. The total recovery of enzyme was 87%. Enzyme activity was measured under standard assay conditions. Top: electrophoretic mobility in a gradient (5–15%) of acrylamide in the presence of $NaDodSO_4$ of the proteins eluted from the column. The gel on the left contained 5 μL (6×10^{-3} A_{280} unit) of the protein solution applied to the column (fraction IV). The gels on the right contained aliquots of the column fractions as shown in the figure: tube 42, 10 μL; tube 46, 10 μL; tube 52, 20 μL; tube 55, 20 μL; tube 60, 20 μL. The mobilities of proteins with known molecular weights are shown in the figure (top left).

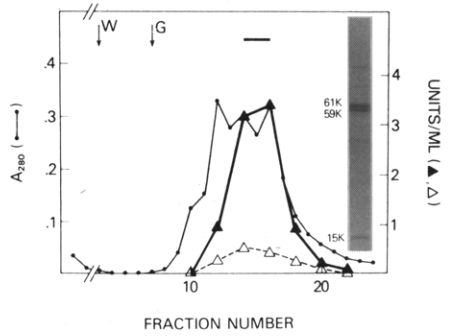

FIGURE 2: Hydroxylapatite chromatography. Fraction V was subjected to hydroxylapatite chromatography, as described in Materials and Methods. During loading, 6-mL fractions were collected. Fractions of 0.85 mL were collected during washing of the column with the equilibration buffer (W) or with the potassium phosphate gradient (G), as shown by the arrows. Activity was measured under standard conditions in the presence of 10^{-6} M CDR (▲) or in the absence of CDR and in the presence of EGTA (△). The overall recovery of enzyme units was 70%. Only fractions 16–18 were pooled, as shown on the figure, and subjected to the next purification step. The insert shows the gel electrophoretic pattern of the pooled fractions (16–18) in the presence of $NaDodSO_4$ (9 μg). The molecular weights of the major components are indicated on the figure.

of equilibrating buffer and eluted with a linear gradient (0.01–0.3 M) of potassium phosphate buffer (pH 7.8) containing 0.1 M NaCl, 0.1 mM dithioerythritol, and 20% glycerol. The total volume of the gradient was 60 mL. Fractions of 0.85 mL were collected in tubes containing enough $MgCl_2$ to bring the final concentration to 1 mM. Although this step did not result in a significant increase in specific activity, probably because of the instability of the enzyme after the gel filtration step, the protein elution profile (Figure 2) indicates that some UV-absorbing material devoid of enzyme activity was resolved from the enzyme. $NaDodSO_4$ electrophoresis analysis of the column fractions (data not shown) showed that proteins composed of large polypeptides (150 000 and 230 000 mol wt) were eluted in fractions 18–20 after the enzyme peak. The two subunits of the inhibitory protein, eluted before the enzyme (tube 12), were also major components of the enzyme fraction (tubes 14–16, fraction VI; insert, Figure 2). Hydroxylapatite chromatography was a convenient step to concentrate and stabilize the enzyme. Fraction VI was stable overnight at 0–4 °C, could be dialyzed against buffer A made 50% in glycerol, and could be stored at −70 °C for up to 3 months without significant loss of activity.

Rechromatography on Sephadex G-200 Superfine. Since the enzyme purified by hydroxylapatite chromatography was consistently contaminated by the inhibitory protein, it was usually subjected to a second gel filtration (Sephadex G-200) which served to resolve this protein (Figure 1). The Sephadex G-200 column (0.9 × 53 cm) was equilibrated with buffer A and was eluted at a flow rate of 0.6 mL/h. The fraction size was 0.9 mL. The enzyme was eluted between fractions 16 and 18. With the exception of the sides of the enzyme peak a good correlation between absorption at 280 nm and enzyme activity was observed (fractions 16–18). As indicated below, the 3–6-fold stimulation of activity by CDR can be increased to 8–10-fold where assays are done in the presence of NH_4Cl. $NaDodSO_4$ gel electrophoresis indicated that, although free inhibitory protein, composed of the 61 000 and 15 000 mol wt subunits, was eluted after the enzyme (tubes 19–22), the two subunits of this protein were also present in tubes 14–18 (data not shown). The fractions with constant specific activity (tubes 16–18) were pooled and stored in small aliquots as described for the enzyme purified up to the hydroxylapatite step. Enzyme preparations purified as described above were stable under these conditions for up to 3 months at −70 °C and were used for all the experiments. A summary of the purification procedure is shown in Table I.

Comments on the Purification Procedure. The overall purification from crude extract was 3000-fold when the specific activity was based on protein measured by absorbance at 280 nm and 2000-fold if the protein content was measured by the method of Lowry. The exact recovery of enzyme activity is difficult to assess for many reasons. Because the enzyme activity in the homogenate is only partly soluble, 30–50% of the enzyme units are lost in the low-speed precipitate. Extraction in buffers containing 0.5 M NaCl gave a slightly better solubilization (60–80% of the units were soluble) but also increased the solubilization of high molecular weight proteins that render subsequent purification steps more difficult. The small (threefold) stimulation of activity by Ca^{2+} observed in crude extracts (Table I) also indicates that a large proportion of the enzyme is not Ca^{2+} dependent. Indeed, the CDR-independent enzyme eluted from the DEAE-cellulose column at higher $(NH_4)_2SO_4$ concentrations than did the Ca^{2+}-dependent phosphodiesterase, explaining the low yield of enzyme after DEAE-cellulose chromatography and the increase in stimulation by Ca^{2+} (six- to tenfold). As will be shown below, it became clear during the purification procedure that the optimal assay conditions were different at different stages of purification. NH_4Cl had only a small 1.5- to 2-fold stimulatory effect in crude extracts in contrast to a three- to fourfold stimulation after the DEAE-cellulose step. The apparent K_m

Table I: Purification of Ca^{2+}-Dependent cAMP Phosphodiesterase[a]

purification steps	vol (mL)	total protein (A_{280} units)	act. (units)[c]	sp act. (units/A_{280} unit)	stimulation[b]	yield (%)
homogenate	2000	nd	800	nd		
I. crude extract[d]	1920	49 000	450	0.009	3.4 (1)	100
II. $(NH_4)_2SO_4$, 30–60%	350	8 960	290	0.03	3.2 (0.8)	64
III. DEAE-cellulose	230	900	117	0.13	6.0[e]	26
IV. activator-Sepharose	11.4	12.9	45	3.50	9.0	10
V. Sephadex G-200	11.2	1.6	21	13.40	5.0	4.7
VI. hydroxylapatite	1.5	0.7	10	14.0	9.0	2.2
VII. Sephadex G-200	1.25	0.2	6.4	30.0	3.6	1.4

[a] The data are based on 500 g of brain. [b] Ratio of Ca^{2+}-stimulated activity (10^{-6} M CDR and 0.05 mM $CaCl_2$) to basal activity (0.025 mM EGTA). The ratios shown in parentheses (basal activity was measured in the presence of 0.05 mM $CaCl_2$ but in the absence of added CDR) indicate CDR dependence. [c] μmol/min under the standard assay conditions. [d] With 8000-rpm supernatant. [e] After the DEAE-cellulose step, the basal activities measured in the presence of EGTA or in the presence of Ca^{2+} but without CDR were not significantly different; Ca^{2+} and CDR dependence were identical.

value of the enzyme for cAMP also increased during purification. The enzyme is now routinely assayed during the purification procedure at 10^{-3} M cAMP in the presence of 0.2 M NH_4Cl. Under these conditions a specific activity of 80–100 units/mg and a 10–13-fold stimulation by CDR are obtained routinely for fraction VI. Some large losses of enzyme activity are not yet understood, such as the 30–40% loss of activity observed during the 30–60% ammonium sulfate fractionation. Despite the fact that all the units were recovered in the 30% $(NH_4)_2SO_4$ supernatant or in 0–50% $(NH_4)_2SO_4$ pellet, a reproducible loss of activity was always observed in the 30–60% $(NH_4)_2SO_4$ precipitation. Addition of the 0–30% $(NH_4)_2SO_4$ fraction did not restore the enzyme units. During the late stages of the purification, the recovery of enzyme from the affinity column could be increased to 70% by using a larger amount of enzyme for the same column, but then we often observed large, irreversible, losses of activity at the subsequent steps, always accompanied by a loss of Ca^{2+} or CDR dependence. These losses could be a consequence of contamination by proteolytic activity or of the low protein concentrations (less than 0.1 mg/mL).

Enzyme Characterization

Gel Electrophoretic Properties. Upon electrophoresis under nondenaturing conditions the purified enzyme exhibited a major broad protein band (Figure 3, gel A) which corresponds to the peak of enzyme activity recovered from a gel run under similar conditions (Figure 3, bottom). Low levels of activity (20% of the units recovered) were distributed between the origin and the enzyme peak associated with the faint protein staining detected above the main band (gel A).[4] A similar pattern was obtained for cGMP hydrolysis (data not shown). Under denaturing conditions the enzyme was shown to consist of three polypeptides with apparent molecular weights of 61 000, 59 000, and 15 000, respectively (Figure 3, gel B). The 61 000 and 15 000 mol wt subunits have the same size as the subunits of the inhibitory protein previously described in this laboratory (Klee & Krinks, 1978). The identities of the 61 000 mol wt subunits of the enzyme and the inhibitory protein were also tested by two-dimensional gel electrophoresis (isoelectric focusing in urea, followed by electrophoresis in $NaDodSO_4$). Both proteins had an isoelectric point of 5.5.

The protein, eluted from the gel run under nondenaturing conditions, was subjected to gel electrophoresis in the presence of $NaDodSO_4$ (Figure 3, gels C and D). Fractions corresponding to the top half of the enzyme peak (fractions 16 and 17, gel C) contained a single protein band with a molecular weight of 59 000.[5] The bottom half of the enzyme peak

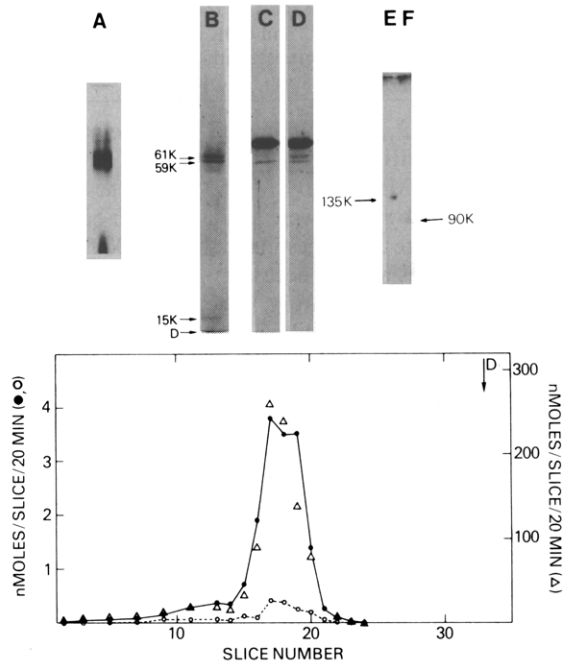

FIGURE 3: Gel electrophoretic properties of CDR-dependent cAMP phosphodiesterase. Fraction VII (9 μg) was electrophoresed in a 5.8% acrylamide gel under nondenaturing conditions. The enzyme was eluted from the sliced gel as described in Materials and Methods and assayed for phosphodiesterase activity in the presence of 0.05 mM Ca^{2+} and 10^{-6} M CDR, at 2×10^{-4} M cAMP (△) or at 10^{-6} M cAMP (●). The activity was also tested in the presence of 0.025 mM EGTA and 10^{-6} M cAMP (○). The overall recovery of enzyme units was 48%. The arrow indicates the position of the tracking dye. (Gel A) Electrophoretic pattern of fraction VII (8.5 μg) electrophoresed under nondenaturing conditions (6.2% acrylamide gel) and stained for protein (the dark area at the bottom of the gel is due to incomplete destaining of the gel). (Gels B–D) $NaDodSO_4$ electrophoretic patterns: (gel B) fraction VII (1.5 μg or 16×10^{-3} unit); (gel C) pooled fractions 16 and 17 eluted from the gel described in the figure (9.0×10^{-3} unit); (gel D) pooled fractions 18 and 19 from the same gel (9.5×10^{-3} unit). The numbers on the left indicate the molecular weights of the major components. The dark protein bands seen across gels C and D correspond to the carrier bovine serum albumin. (Gels E and F) Electrophoretic patterns after electrophoresis in 4–30% gradients of acrylamide run under nondenaturing conditions. (Gel E) Fraction VII (6.5 μg); (gel F) inhibitory protein (3.5 μg). The estimated molecular weights are indicated in the figure.

[4] The low levels of activity between the origin and the enzyme peak were more easily detected by incubating gel slices directly into the incubation mixture as described in Materials and Methods.

[5] Failure to detect the 15 000 mol wt subunit could be the result of its poor staining intensity. When higher protein concentrations were used, the small subunit could be detected, but only associated with the 61 000 mol wt polypeptide.

Table II: Effect of Various Additions on CDR-Dependent cAMP Phosphodiesterase

additions to incubation mixture[a]	enzyme act. (units/mL) +CDR	enzyme act. (units/mL) +EGTA	stimulation[b] (fold)
none	1.9	0.55	3.5
NH_4Cl, 0.2 M	4.5 (+137)[c]	0.43 (−22)	10.5
glycerol, 5%	1.7 (−10)	0.46 (−16)	3.7
$MnCl_2$, 0.07 mM	2.4 (+26)	0.53 (−4)	4.5
imidazole, 0.05 M	3.4 (+79)	0.59 (+7)	5.8
NH_4Cl + glycerol	4.6 (+142)	0.50 (−10)	9.2
NH_4Cl + imidazole	4.5 (+137)	0.40 (−23)	11.3
imidazole + glycerol	3.0 (+58)	0.57 (+4)	5.3
NH_4Cl + imidazole + glycerol	3.9 (+105)	0.40 (−27)	9.8

[a] The incubation mixtures contained 10^{-3} M cAMP, 10^{-6} M CDR, and 0.05 mM $CaCl_2$ or 10^{-3} M cAMP and 0.025 mM EGTA. [b] The stimulation by CDR is the ratio of the enzyme activities measured in the presence of Ca^{2+} and CDR and in the presence of EGTA. [c] The numbers in parentheses indicate the percent inhibition or activation by the various additions.

Table III: Effect of cAMP, NH_4Cl, and NaCl on the Apparent K_m Values of cAMP Phosphodiesterase for CDR

assays conditions[a] additions	cAMP (M)	app K_m[b] (M)
none	10^{-3}	$1.4 \pm 0.4 \times 10^{-9}$ (3)[c]
none	10^{-6}	$3.5 \pm 0.5 \times 10^{-9}$ (2)
NaCl, 0.2 M	10^{-3}	$1.3 \pm 0.6 \times 10^{-9}$ (3)
NaCl, 0.2 M	10^{-6}	$3.9 \pm 0.5 \times 10^{-9}$ (2)
NH_4Cl, 0.2 M	10^{-3}	$1.6 \pm 0.3 \times 10^{-9}$ (3)
NH_4Cl, 0.2 M	10^{-6}	$5.4 \pm 0.2 \times 10^{-9}$ (2)

[a] Assays conditions were as described in Materials and Methods, the enzyme concentration was $12–18 \times 10^{-3}$ unit/mL and the incubation time varied between 5 and 20 min. [b] The apparent K_m is the concentration of CDR needed to obtain half-maximum stimulation of the enzyme. Similar K_m values were obtained by double-reciprocal plots. [c] Numbers in parentheses indicate the number of determinations.

(fractions 18 and 19, gel D) contained the three polypeptides detected in the purified enzyme. The enzyme activity measured at 10^{-3} M cAMP and 10^{-6} M cAMP and the stimulation of enzyme activity by CDR were similar in both halves of the enzyme peak (Figure 3), suggesting that the 61 000 and 15 000 mol wt polypeptides are not necessary for phosphodiesterase activity or for CDR stimulation. The apparent coincidence of enzyme activity and inhibitory protein after gel electrophoresis could result from a very similar electrophoretic behavior of the enzyme and its inhibitor. Although Ferguson plot analysis of the enzyme protein and the inhibitory protein yielded different molecular sizes, 135 000 and 90 000, respectively (bovine serum albumin and its dimer were used as standards), their free electrophoretic mobilities were such that no appreciable separation of the two proteins could be achieved by electrophoresis in 5–8.5% acrylamide gels.

The different sizes of the two proteins were confirmed by electrophoresis in a 4–30% gradient of acrylamide (Pharmacia) which gave a clear fractionation on the basis of size. Purified inhibitory protein exhibited a single protein band of 90 000 mol wt (Figure 3, gel F). Fraction VII contained a major protein band (130 000 mol wt) and no detectable protein corresponding to a mol wt of 90 000 (Figure 3, gel E). The lack of fractionation of the enzyme and the inhibitory protein in the gradient of acrylamide could indicate interaction between the two proteins and would therefore explain the difficulties encountered in attempting to fractionate them.

Catalytic Properties. The specific activity of the purified enzyme under our standard assay conditions was 30 units/mg. Under optimal conditions the specific activity was 80–100 units/mg. The pH dependence of enzyme activity was similar to that of the crude enzyme described by Butcher & Sutherland (1962). The pH optimum was 8.0 in Tris-HCl or Hepes buffers. Under these conditions the reaction rate was linear, as a function of time (2–60 min) and enzyme concentration, up to 15% of substrate utilized. The purified enzyme hydrolyzed 10^{-3} M cGMP and 10^{-3} M cAMP at similar rates. At low substrate concentration (10^{-6} M), cGMP was hydrolyzed much faster than cAMP [the K_m for cGMP was $(2–5) \times 10^{-6}$ M]. cGMP was a competitive inhibitor of cAMP hydrolysis with a K_i of 1.6×10^{-5} M.

In view of the diverse assay conditions used in different laboratories, the effects of various additions to the incubation mixture on the rate of cAMP hydrolysis were tested (Table II). Glycerol and $MnCl_2$ had marginal effects on the CDR-dependent activity. Imidazole and NH_4Cl were the only potent activators of the enzyme, NH_4Cl being reproducibly more active. The effects of these two compounds were not additive; on the contrary, a slight inhibition was observed upon addition of imidazole and NH_4Cl to the same incubation mixture. Since the basal activity of the enzyme (in the presence of EGTA) was not significantly affected by imidazole or NH_4Cl, an apparent increase in CDR stimulation was observed.

The stimulation of phosphodiesterase by imidazole originally described by Butcher & Sutherland (1962) has been studied in several laboratories (Wells et al., 1975; Ho et al., 1976; Donnelly, 1976). The effect of imidazole on the purified enzyme is dependent on the assay conditions. At high concentration of cAMP (10^{-3} M), imidazole up to 50 mM stimulated both CDR-stimulated and basal activities. Higher concentrations of imidazole always resulted in inhibition of cAMP hydrolysis. At low cAMP concentration (10^{-6} M) imidazole had a pronounced inhibitory effect on CDR-stimulated activity and no significant effect on basal activity. At similar concentrations, NaCl had a negligible inhibitory effect on enzyme activity.

Stimulation of the enzyme by NH_4^+ was first reported for the heart enzyme by Nair (1966) and is responsible for the apparent increased activity sometimes observed in the course of our purification after ammonium sulfate fractionation steps. The stimulation specific for NH_4^+ ions was not observed with Na_2SO_4, NaCl, or KCl and is therefore not an effect of ionic strength. The effect of NH_4Cl was very similar to that of imidazole but, in the presence of 10^{-6} M cAMP, NH_4Cl had a small stimulatory effect on CDR-dependent activity in contrast to the large inhibitory effect observed with imidazole.

Stimulation of cAMP Phosphodiesterase by CDR. To characterize further the effects of imidazole or NH_4Cl on enzyme activity, we studied first the catalytic properties of the enzyme in the presence and absence of CDR (in the absence of imidazole or NH_4Cl). Since it was reported previously that cyclic nucleotide levels affect the affinity of the enzyme for CDR (Teo et al., 1973; Wolff & Brostrom, 1974), the stimulation by CDR was measured in the presence of 10^{-3} and 10^{-6} M cAMP. The percent stimulation measured at 10^{-9} M CDR and 10^{-3} M cAMP was independent of enzyme concentration ($0.03–0.5 \times 10^{-3}$ A_{280} unit/mL). The K_m values summarized in Table III can therefore be taken as an approximation of the dissociation constants. These data also indicated that a significant proportion of the enzyme protein did not interact with CDR and could represent inactive enzyme (with fresh enzyme, at concentrations of $0.07–2 \times 10^{-3}$ A_{280} unit/mL, a strong enzyme concentration dependence of the

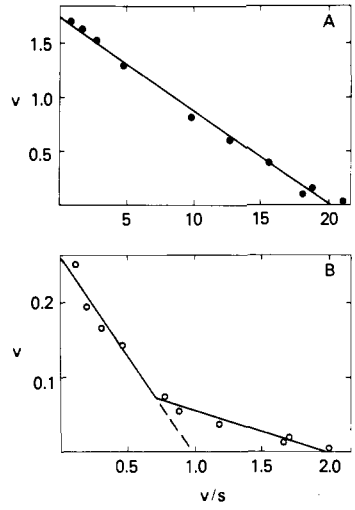

FIGURE 4: Eadie plot of basal and CDR-stimulated activities. The enzyme was assayed as described in Materials and Methods. (A) In the presence of 0.05 mM Ca^{2+} and 10^{-6} M CDR (●) or (B) in the presence of 0.025 mM EGTA (○). The velocity is expressed as μmol/(min·mL of enzyme) and the substrate concentration in μmol/mL (other preparations of enzyme contained less of the low K_m component than that analyzed in B).

Table IV: Effect of CDR, NH_4Cl, and Imidazole on the Kinetic Constants of Phosphodiesterase[a]

additions	V_m (units/mL of enzyme)		K_m (mM)	
	−CDR[b]	+CDR	−CDR	+CDR
none	0.26 ± 0.02	1.56 ± 0.07	0.31 ± 0.07	0.1 ± 0.02
0.2 M NH_4Cl	0.84 ± 0.02	5.80 ± 0.16	0.41 ± 0.02	0.22 ± 0.02
0.1 M imidazole[c]	0.76 ± 0.16	4.42 ± 0.14	1.90 ± 0.60	0.75 ± 0.05

[a] The kinetic constants were determined as described in Materials and Methods. The K_m values in the absence of CDR determined by this method represent the high K_m values determined by the Eadie plot since the computer analysis of the data maximizes the contribution of the determinations done at high cAMP concentrations. [b] Experiments were done in the presence of 10^{-6} M CDR (+CDR) or in the presence of 0.025 mM EGTA (−CDR). [c] The concentration of CDR was 2×10^{-6} M.

apparent K_m was observed). As shown in Table III (lines 1 and 2), the K_m of the enzyme for CDR was decreased two- to threefold when 10^{-3} M cAMP was present in the assay as opposed to 10^{-6} M cAMP.

Having determined conditions under which the enzyme is saturated with CDR, we were able to analyze the kinetic parameters of the enzyme in the presence of EGTA (basal activity) and in the presence of Ca^{2+} and CDR (stimulated activity). The crude enzyme (fraction III) exhibits anomalous kinetics indicative of negative cooperativity or multiple enzymatic species under both conditions.[6] The purified enzyme, however, follows Michaelis–Menten kinetics in the presence of Ca^{2+} and CDR (Figure 4A). In the presence of EGTA, the Eadie plots were often not linear (Figure 4B). The Hill coefficients determined in the presence of CDR and EGTA were 1.0 and 0.83, respectively. As summarized in Table IV (line 1), the enzyme has an apparent K_m for cAMP of 0.31 mM in the presence of EGTA. Addition of CDR and $CaCl_2$ resulted in a decrease of the K_m value to 0.10 mM and an increase in the V_m value from 0.26 to 1.56 units/mL. Addition of NaCl (0.2 M) had no significant effect on the kinetic constants.

[6] C. B. Klee, unpublished observations.

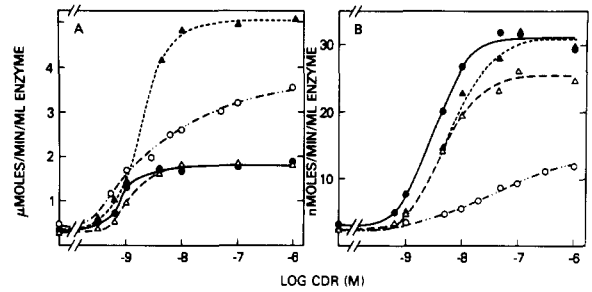

FIGURE 5: CDR concentration dependence of cAMP phosphodiesterase activation. Fraction VII was assayed as described in Materials and Methods and Table IV. (A) The cAMP concentration was 10^{-3} M; (B) 10^{-6} M cAMP. (●) No addition; (△) 0.2 M NaCl added; (▲) 0.2 M NH_4Cl added; (○) 20 mM imidazole added.

Stimulation of cAMP Phosphodiesterase by NH_4Cl and Imidazole. The effect of NH_4Cl and imidazole on the dependence of enzyme stimulation on CDR concentration is shown in Figure 5. Addition of NH_4Cl had a small inhibitory effect on the affinity of the enzyme for CDR similar to that observed upon addition of NaCl (Table III). NH_4Cl increased the extent of stimulation by CDR three- to fourfold, but only at 10^{-3} M cAMP (Figure 5A). Stimulation was not observed at 10^{-6} M cAMP (Figure 5B). In contrast to NH_4Cl, imidazole affected both the extent of stimulation and the apparent K_m value of the enzyme for CDR. As shown in Figure 5, in 50 mM imidazole the affinity of the enzyme for CDR was greatly reduced and the activation curves suggest negative cooperativity. The reduction in enzyme affinity for CDR prevented full activation of the enzyme by imidazole at 10^{-3} M cAMP (Figure 5A) and resulted in apparent inhibition of stimulation by CDR at 10^{-6} M cAMP (Figure 5B). Saturating levels of CDR were not obtained even at 10^{-6} M CDR.

As anticipated from the experiments described above, NH_4Cl greatly increased the rate of hydrolysis of cAMP by increasing the V_m value to 5.8 ± 0.16 units/mL (in the presence of CDR) and to 0.84 ± 0.02 unit/mL (in the presence of EGTA) at its optimal concentration (0.2 M) (Table IV). In contrast to CDR, which increases V_m and decreases K_m, NH_4Cl increased both V_m and K_m (Table IV). The small effect of NH_4Cl on the K_m for cAMP may explain the lack of NH_4Cl stimulation observed in the presence of EGTA (Table II), since these experiments were not performed at saturating substrate concentrations and since the K_m value of the enzyme for cAMP is lower in the presence of $CaCl_2$ than in the presence of EGTA. It should be noted that NH_4Cl exerts its effect both on the basal and the stimulated activities and therefore does not only modify the enzyme–CDR interaction. The ability of imidazole to increase the V_m values of the enzyme is similar to that of NH_4Cl (Table IV), although lower concentrations of imidazole than NH_4Cl were required to activate the enzyme. Imidazole at high concentrations was strongly inhibitory (data not shown), probably because of its inhibitory effect on CDR binding to the enzyme (Figure 5A,B). In contrast to NH_4Cl, which had only a small effect on the K_m values for cAMP, imidazole increased the K_m values dramatically both in the presence and absence of CDR (Table IV). The combined effects of imidazole on the affinities of the enzyme for CDR and cAMP are responsible for the inhibition of cAMP hydrolysis at very low concentrations of substrate.

Discussion

The Ca^{2+}-dependent cAMP phosphodiesterase has been purified to apparent electrophoretic homogeneity. Its mo-

lecular weight was estimated by the Ferguson plot analysis, electrophoresis in gradients of acrylamide, and gel filtration to be 135 000 ± 10 000. Its sedimentation coefficient in glycerol gradients is 5.75 (Richman & Klee, 1979). These values agree well with those determined for the partially purified brain enzyme (Lin et al., 1975; Kakiuchi et al., 1975; Sakai et al., 1977) and the purified heart enzyme (Ho et al., 1977). Gel electrophoresis under denaturing conditions revealed three polypeptides of molecular weights 61 000, 59 000, and 15 000. A CDR-binding protein which has been described in brain extracts by Wang & Desai (1977) and purified by affinity chromatography in this laboratory consists of two subunits of molecular weights 61 000 and 15 000 (Klee, 1977a; Klee & Krinks, 1978). This protein is referred to as "inhibitory protein" because of its ability to inhibit phosphodiesterase activation by CDR. As shown above, the 61 000 mol wt subunits of the enzyme and the inhibitory protein also have the same isoelectric point and are perhaps identical. Although the two proteins may share this subunit, we were unable to show that the 61 000 mol wt chain is necessary for hydrolysis of cAMP or cGMP or for CDR stimulation. Cross-linking of the enzyme with radioactive CDR indicated that only the two large polypeptides interact with CDR; no labeled 32 000 mol wt species (complex of the 15 000 subunit and CDR) was detected by gel electrophoresis in $NaDodSO_4$ after cross-linking with dimethyl suberimidate. The three-subunit enzyme described in Table I has a specific activity, under optimal conditions, of 80–100 units/mg. It contains similar amounts of the two large subunits as judged by their staining intensities. We occasionally obtained CDR-dependent preparations with higher specific activities (150–350 units/mg) that contained less of the 61 000 mol wt subunit; the low yield and the lack of stability of these preparations prevented further study. The catalytic activity may therefore be assigned to the 59 000 mol wt subunit since the other two components by themselves are devoid of activity. The failure to separate the enzyme from its inhibitor (which has a different molecular weight) by repeated gel filtrations and chromatography may indicate that the two proteins interact physically. The presence of inhibitory protein on both sides of the enzyme peak on the second Sephadex G-200 chromatography is consistent with this hypothesis. Phosphodiesterase, with an apparent molecular weight of 135 000, could be a complex of the 59 000 mol wt subunit interacting with the inhibitory protein. Brain Ca^{2+}-dependent phosphodiesterase is clearly different from the homogeneous cGMP phosphodiesterase purified from rod outer segments of frog retina which is not stimulated by Ca^{2+} (Miki et al., 1975) and has subunit molecular weights of 120 000 and 110 000. The subunit size of the Baker's yeast enzyme (Fujimoto et al., 1974) and the molecular weight of one purified bacterial enzyme (Okabayashi & Ide, 1970) are very similar to that of the brain phosphodiesterase.

The Ca^{2+}-dependent phosphodiesterase exhibits the kinetic behavior ascribed previously to "the high K_m" enzyme (for a recent review, see Wells & Hardman, 1977). As reported for less purified preparations, we show that the 59 000 mol wt subunit can catalyze the hydrolysis of both cAMP and cGMP in a Ca^{2+}-dependent mechanism. The enzyme activity is greatly affected by the exogenous ligands, imidazole and NH_4Cl. The physiological significance of these ligands is not clear at present but a careful study of their effects may help to elucidate the mechanism of the reaction and of its regulation. Omission or addition of these compounds to assays may be responsible for some of the conflicting results reported in the literature. NH_4Cl stimulates both basal and CDR-stimulated phosphodiesterase by interacting with the enzyme and the enzyme–substrate complex; it increases both V_m and K_m (the effect on K_m prevents stimulation by NH_4Cl at low cAMP concentrations). The small decrease in the affinity of the enzyme for CDR observed in the presence of NH_4Cl is probably due to increased ionic strength. Imidazole also increases V_m and K_m of the enzyme for cAMP, but its effect on K_m is larger than that of NH_4Cl (Table IV). In addition, imidazole strongly inhibits the interaction of the enzyme with CDR (Figure 5). This effect of imidazole may be responsible for the dissociation of CDR from the erythrocyte membrane Ca^{2+}-dependent ATPase observed by Farrance & Vincenzi (1977). In view of these complex effects of imidazole or NH_4Cl, the stimulation of the enzyme by CDR is best analyzed in the absence of these ligands.

The activation of the enzyme by CDR is the result of a large increase (eight- to tenfold) in the V_m value and a twofold decrease in the apparent K_m value, as had previously been observed with partially purified enzymes (Teo & Wang, 1973; Cheung, 1971; Kakiuchi et al., 1973; Filburn et al., 1978). Notably, however, in the presence of EGTA, the enzyme exhibits negative homotropic kinetics. This conversion from negative cooperativity to Michaelis–Menten type kinetics upon addition of CDR was also reported by Donnelly (1977) for the hydrolysis of cGMP by the bovine heart phosphodiesterase. Negative cooperativity has been attributed to the existence of multiple enzymatic species or to ligand-induced conformational changes (Levitzki & Koshland, 1969). A slow isomerization between two enzymatic forms with different kinetic properties according to Ainslie et al. (1972) was ruled out on the basis of the linearity of the time course experiments. It is also unlikely that contamination of the enzyme by CDR is responsible for the abnormal kinetics since the experiments were carried out in the presence of EGTA. The presence of a small amount of partially proteolyzed enzyme which is known to be CDR independent and in an activated state (Cheung, 1967; Sakai et al., 1977) could explain the abnormal kinetics observed in the presence of EGTA. Addition of CDR, which interacts only with the undegraded enzyme and increases its V_m and decreases its K_m, could lead to apparent homogeneity and linear Michaelis–Menten type kinetics. The slight decrease in K_m value for cAMP, from 0.30 to 0.1 mM, is best explained by a higher affinity of the CDR–enzyme complex for cAMP in view of the ability of the substrate to decrease the apparent K_m of the enzyme for CDR.

Alternatively, the negative cooperativity of the enzyme could be the result of the existence of two interconvertible enzymatic species with different affinities for cAMP and for CDR. Assuming that CDR interacts only with one species of the enzyme (CDR-dependent form), CDR would shift the equilibrium in favor of this enzymatic species and the kinetics would become linear. The effect of cAMP on the K_m for CDR, in this case, could simply be the result of the cAMP-induced conversion to the CDR-dependent enzyme. This model could also account for the greater extent of CDR stimulation observed at 10^{-6} M cAMP than at 10^{-3} M cAMP if the "high affinity" CDR-independent form of the enzyme had a lower V_m than the "low affinity" CDR-dependent enzyme. According to this model, the enzyme would adapt its catalytic capacity to physiological levels of cAMP. The concerted regulation by multiple ligands such as cAMP, CDR, and Ca^{2+} permits a very precise modulation of enzyme activity over a wide range of substrate concentrations. Although the high K_m of this enzyme makes it unlikely that the enzyme acts at physiological cAMP concentrations, temporary, localized,

increases of cAMP concentrations could occur in the cell and such events could be controlled by the "high K_m" enzyme.

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