Purification and Properties of a Cyclic-AMP Phosphodiesterase That Is Active in Only One Cell Type during the Multicellular Development of *Dictyostelium discoideum*†

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ABSTRACT: Cyclic-AMP phosphodiesterase (PDE) accumulates during the aggregation stage of *Dictyostelium* where it functions in maintaining extracellular levels of cyclic AMP (cAMP). The activity decreases during the subsequent multicellular slug stage and then accumulates again during sorocarp construction, but the enzyme is active only in the developing stalk. Because of the possible significance of this localized activity in only one of the two cell types, we have purified the enzyme from the multicellular stage in order to understand its mode of regulation in vivo. We find that the enzyme which is localized in the prestalk cells is similar in many respects to the extracellular PDE which is active at the aggregation stage. The enzyme from both stages is inhibited by a low molecular weight protein. The mechanism of this inhibition is through a shift in the apparent K_m for cAMP from micromolar to millimolar levels. The inhibited form of the enzyme can be activated by preincubation with $MgSO_4$ and dithiothreitol (DTT). This activation treatment releases the inhibitor from the enzyme, thus restoring the low K_m form, changes the molecular weight of the culmination stage enzyme from 95 000–100 000 to 68 000 by releasing the M_r 35 000–40 000 inhibitor protein, and causes irreversible loss of inhibitor activity. Although the inhibitor could be obtained in high yield from the aggregation stage by simply heating the extracellular fluid, it could not be detected from culmination stage extracts when prepared by this method. However, inclusion of calcium in the extraction buffer resulted in release of inhibitor from both heated and nonheated samples. The results indicate that the stalk cell specific PDE is regulated similarly to the aggregation stage PDE and opens the possibility of differential regulation of PDE in the two cell types.

Removal of a nutrient source from cultures of *Dictyostelium discoideum* amoebae triggers the aggregation of the individual cells and subsequent development of a multicellular fruiting body. The chemotactic signal which directs these cellular movements is adenosine cyclic 3′,5′-phosphate (cAMP) (Konjin et al., 1968). The biochemical processes which underlie the cAMP-directed chemotaxis of the aggregating amoebae have been extensively studied, and it is known that an extracellular cAMP phosphodiesterase (ePDE), 3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase (EC 3.1.4.17), is secreted by the amoebae along with an endogenous inhibitor (PDI). Interaction of the enzyme and its inhibitor presumably maintains a gradient of cAMP which is appropriate as a directional signal for chemotaxis. Until recently, the literature on the extracellular PDE presented a rather confusing picture of many kinetically distinct, possibly interconvertible forms and a specific inhibitor protein which had not been extensively characterized (Chang, 1968; Chassy, 1972; Pannbacker & Bravard, 1972; Riedel et al., 1972; Dicou & Brachet, 1979; Toorchen & Henderson, 1979). Kessin et al. (1979) have recently resolved some of the confusion in the earlier literature by demonstrating the mechanism of the interaction between the PDI and the extracellular enzyme.

While PDE is known to be active in the pseudoplasmodium (slug) and later fruiting stages of *Dictyostelium* (Malkinson & Ashworth, 1973; Brenner, 1978), it has not been extensively studied from these stages. The activity was generally considered to remain unchanged throughout development (Nestle & Sussman, 1972). We have followed the activity of PDE through all stages of the developmental cycle and have found that the activity peaked at the aggregation stage and then decreased during the multicellular slug stage (Brown & Rutherford, 1980). Upon subsequent differentiation of the two cell types, stalk and spore cells, PDE activity reappeared in developing prestalk cells but was not detectable in prespore cells. However, Mg–dithiothreitol (Mg–DTT) treatment of the prespore extracts resulted in PDE activity that was nearly equal to the stalk levels. Extracts from stalk cells were not affected by the Mg–DTT treatment. Since this activation step is known to release the aggregation stage ePDE from its endogenous inhibitor, we reasoned that the activation observed with the prespore enzyme was also due to the release of an inhibitor and that prespore cells contained a cryptic form of the enzyme. If the distribution of PDE activity in the two cell types represents the activity of PDE in vivo, a potential exists for creating a gradient of cAMP between the two cell types, a result often envisioned by models which attempt to explain cellular patterning during development [for a review, see Rutherford et al. (1982)]. In this report, we have attempted to understand the mechanism of this cell-specific localization of PDE activity by purifying the culmination stage enzyme and characterizing its mode of regulation.

Experimental Procedures

Culture of Dictyostelium discoideum. Growth and differentiation of amoebae of *D. discoideum* were carried out as previously described (Rutherford, 1976). Myxamoebae of the wild-type strain NC4 were grown from spores in two-membered cultures with *Escherichia coli* on sheets of nutrient agar. As amoebae began to clear the culture of bacteria (48 h grown at 23 °C), they were washed off the agar in ice-cold Bonner's salts solution (Bonner, 1947) and collected by centrifugation at 500*g* for 1 min. The amoebal pellet was washed twice in the same solution to remove remaining bacteria and then was spread on 2% nonnutrient agar sheets. At various stages of development, the cells were washed from the nonnutrient agar surface with ice-cold distilled water, pelleted by low-speed centrifugation at 500*g* for 5 min, and frozen at −76 °C. After

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lyophilization, the cells were stored under vacuum at −76 °C. No loss of PDE activity under these storage conditions occurred over a period of 1 year.

Assay of Phosphodiesterase. PDE was assayed according to the method of Thompson et al. (1974), in which degradation of [8-^{3}H]cAMP is coupled to cobra (*Ophiophagus hannah*) venom nucleotidase. The assay was performed in a total volume of 100 μL containing 100 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid buffer, pH 7.5, 1 mM $MgSO_4$, 100 μM 8-^{3}H-labeled cAMP (6×10^9 cpm/mmol), and sufficient enzyme to degrade 5–20% of the total substrate in a 1-h incubation at 25 °C. At the end of 1 h, the tubes were boiled for 1 min and cooled, and a 1-μL sample of 25 mg/mL lyophilized *O. hannah* venom in Hepes, pH 7.5, was added. After a 20-min digestion of the 5′-AMP product by the venom nucleotidase at 25 °C, 0.5 mL of ice-cold Dowex AG1×2 resin (1:4 in 10 mM acetic acid) was added, and the tubes were held (on ice) with occasional mixing for 15 min. The resin was then pelleted by centrifugation at 500*g* for 10 min and 400 μL of the supernatant counted in 3 mL of scintillation fluid. Radioactive nucleotides were obtained from Amersham Searle. Other reagents and coupling enzymes used in the PDE assay were obtained from Sigma Chemical Co., St. Louis, MO. One unit of PDE is defined as the amount which catalyzes the destruction of 1 nmol of cAMP per min at 25 °C.

We found that acetic acid must be added to the resin suspension according to the modification of Boudreau & Drummond (1975) to prevent nonspecific binding to the resin. The optimum concentration of acetic acid in the resin was determined by mixing assay buffer (100 μL) containing authentic cAMP, 5′-AMP, adenosine, or inosine with 0.5 mL of resin suspended in various concentrations of acetic acid. The amount of nucleotide remaining in the supernatant was quantified by the absorption at 259 nm. We found that 10 mM acetic acid gave optimum recovery of adenosine (73%) and inosine (69%) at the concentrations at which they were produced in this assay. Therefore, a "resin recovery" factor of 1.43 was included in all calculations.

The spectrophotometric assay of Bergmeyer (1974) was used for the assay of inhibitor and antibody inhibition of the purified enzyme, and for monitoring activity during isolation of the enzyme. The standard reaction mixture contained 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5, 1 mM $MgSO_4$, 0.1 mg/mL lyophilized *O. hannah* venom, and 0.8 unit/mL adenosine deaminase (Sigma type 1). Reaction mixture (400 μL), enzyme (5–20 μL), and cAMP (1 μL of a 10 mM solution) were rapidly mixed in a quartz semimicro cuvette, and the absorbance was monitored at 265 nM. This assay gave PDE values in close agreement with the radioactive assay.

Purification and Assay of the Extracellular PDE Inhibitor. In the standard assay, 0.2 unit of enzyme was mixed with dilutions of inhibitor, preincubated for 15–20 min at room temperature, and assayed by either the spectrophotometric or the radiometric methods. One unit of inhibitor is defined as the amount necessary to reduce the activity of 2 units of PDE by 50%. Because of the tight binding nature of the inhibitor (Riedel et al., 1972), it was not necessary to control the dilution factor for reproducible results. For assay of inhibitor in column fractions or heated tissue homogenates, the enzyme was added at an activity level which approximately matched the PDE activity of the sample before heating.

The inhibitor was partially purified by a modification of the method of Reidel et al. (1972). About 5 L of the extracellular fluid from the washed cells was brought to 80 °C for 10 min to inactivate extracellular PDE and dissociated inhibitor-bound PDE. The solution (pH 4.9) was then acidified to pH 3.3 with concentrated HCl and stirred at room temperature for 30 min. The resulting precipitate was removed by centrifugation at 217000*g* for 30 min. The supernatant was neutralized with 10 M NaOH, and the heavy precipitate which formed was also removed by centrifugation. The extract was then brought to 80% saturation with solid ammonium sulfate (stirred at room temperature 1 h), and the resulting precipitate was resuspended in 10 mM Tris-HCl buffer, pH 7.5, and then dialyzed overnight against the same buffer. The concentrated protein fraction was brought to 50 mM Tris-HCl, pH 7.5, and applied to diethylaminoethylcellulose (DEAE-cellulose) (Whatman DE52, 50-mL bed volume) in the same buffer. The inhibitor was completely retained on the column at this pH and could be eluted with a 0–0.5 M NaCl gradient at approximately 0.1 M NaCl. The active fractions were pooled, concentrated by ammonium sulfate precipitation as described above, and applied to a Sepharose 6B column. The active fractions collected from this column were concentrated by ultrafiltration on an Amicon PM 10 membrane and stored frozen at −76 °C.

Immunochemistry. Purified soluble PDE from five purification runs (10 g of lyophilized mass culture in the culmination stage) was concentrated by ultrafiltration into 0.5–0.8 mL of H_2O, emulsified with an equal volume of Freunds adjuvant, and injected intramuscularly in a New Zealand white rabbit. A 10–15-mL sample of serum was collected from the marginal ear vein prior to each injection for assay of anti-PDE activity. The rabbit was exsanguinated when the titer remained unchanged for 3 weeks. Serum samples were heated at 56 °C for 30 min to inactivate complement and then centrifuged at 15000*g* for 30 min. The heated serum was precipitated with ammonium sulfate at 50% saturation and then was dialyzed overnight against 15 mM potassium phosphate buffer, pH 8.0 at 8 °C. The dialyzed sample was then applied to a 35-mL bed volume of DEAE-cellulose (Whatman DE52) equilibrated in the same buffer (25 mg of serum protein/mL of resin). The flow-through volume containing the immunoglobulin G (IgG) fraction was concentrated by ultrafiltration on an Amicon YM 10 membrane and stored frozen at −76 °C.

Isoelectric Focusing in Granulated Gel. Preparative isoelectric focusing of the DEAE pH 8.5 flow-through enzyme was carried out on an LKB 2117 Multiphor flatbed electrophoresis unit by using an LKB Model 2103 power supply. Gels were prepared according to Winter et al. (1975). The enzyme was concentrated by ultrafiltration into 25 mL of 5 mM Tris, pH 8.5, and cast into 100 mL of 6% Ultrodex (LKB) gel (12 × 25 cm) containing 2% ampholines, pH range 7–11. After being focused at 8-W constant power for 15 h, a fractionating grid with 0.7 × 11 cm sections was pressed into the gel, and the sections were tested for pH with an M1-410 microelectrode (Microelectrodes, Inc.). Then their contents were scraped out and eluted with 1 mL of 25 mM Tris-HCl, pH 8.5, for assay of PDE activity. With adequate cooling during the focusing run, recovery of enzyme activity was at least 80% of the applied units.

Results

Purification Procedure. Homogenates were prepared by passing the cells through a French pressure cell at 15000–20000 psi or by freezing and subsequent lyophilization. The lyophilized tissue was suspended in 25 mM Tris-HCl buffer, pH 8.5, at 20 mg dry weight/mL, ground in a glass homogenizer with a motor-driven Teflon pestle (5 strokes, 150 rpm), and centrifuged at 100000*g* for 60 min. The soluble PDE was precipitated between 30% and 70% ammonium sulfate satu-

Table I: Purification of Soluble Phosphodiesterase

fraction	sp. act[a]	% recovery	x-fold purification
homogenate: lyophilized culms, 20 mg/mL	0.63	100	
8000*g* supernatant	0.49	54	none
100000*g* supernatant	0.49	48	none
30–70% $(NH_4)_2SO_4$	1.2	30	1.9
Mg^{2+}–DTT activation	6.8	88	10.8
DEAE pH 8.5 flow through	45.0	35	70.0
Con A, αMM elution	590.0	25	940.0
other fractions[b]			
8000*g* pellet	1.3	45	
0–35% $(NH_4)_2SO_4$	0.13	1	
DEAE pH 8.5 salt peak	4.7	11	

[a] Specific activity is expressed as units per milligram of protein. One unit of activity is defined as the nanomoles of cAMP hydrolyzed per minute at 25 °C, at a substrate concentration of 100 μM cAMP. [b] These are fractions containing activity but not included in the purification.

ration and then dialyzed into 25 mM Tris-HCl, pH 8.5, containing 0.1 M $MgSO_4$ and 5 mM DTT (Mg–DTT) overnight at 4 °C. This activation treatment resulted in a 5-fold increase in total activity (Table I). The fact that the soluble cell-associated PDE from the culmination stage could be activated by a treatment known to activate the extracellular enzyme from the aggregation stage was early evidence that the two activities were related.

The activated enzyme (usually 12–15 mL from a single isolation from 2 g of lyophilized tissue) was then placed on a column of Sepharose 6B and eluted with 25 mM Tris-HCl. Recovery of activated units was typically 50–60% from the column, the 10% or less which emerged in the high molecular weight void volume being discarded.

The active fractions from the Sepharose chromatography were pooled and slowly siphoned over a column of DEAE-Sephadex A25 in 25 mM Tris-HCl at pH 8.5. From 30% to 40% of the applied activity emerged in the flow through, with a large increase in the specific activity, as most proteins in the sample bound to the resin at this high pH. Additional PDE could also be eluted with a 0–0.5 M KCl gradient. This fraction (eluting at approximately 0.1 M KCl) varied in amount but was occasionally as much as 20–25% of the applied units. This "salt peak" enzyme was not included in the further purification steps; however, it was characterized as to molecular weight and extent of activation. The activity in the flow-through volume was applied to a concanavalin A–Sepharose (Con A–Sepharose) affinity resin. Since the enzyme is inactivated at high dilution, it was advantageous to apply the DEAE flow-through material directly to Con A and apply a salt wash (1 M NaCl) afterward to elute nonspecifically absorbed material. The resin was also washed with 0.3 M galactose prior to methyl α–mannoside elution to remove discoidin, a slime mold lectin which binds to the Sepharose matrix (Simpson et al., 1974). Removal of proteins by the galactose wash was verified by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. The soluble, activated enzyme was quantitatively eluted in 1% methyl α-mannoside. Activated PDE quantitatively eluted from Con A–Sepharose both prior to and after ion-exchange chromatography. This suggested that the two molecular weight forms of the enzyme present in the activated soluble preparations (see below) were both glycoproteins.

The final enzyme preparation obtained from affinity chromatography was purified at least 1000-fold (Table I). Upon

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of PDE. The electrophoresis of the purified enzyme was conducted on 7.5% polyacrylamide tube gels containing 0.13% $NaDodSO_4$. Molecular weight markers were ribonuclease A (M_r 13 700), chymotrypsinogen (M_r 25 000), ovalbumin M_r 45 000), and bovine serum albumin (M_r 67 000).

$NaDodSO_4$ gel electrophoresis, the final preparation revealed a single protein peak at M_r 65 000–68 000 (Figure 1). An M_r 65 000 form is also the smallest PDE reported from isolation of the extracellular enzyme (Toorchen & Henderson, 1979), and it is probable that these are the same protein. The triplet peak, but not the main peak, was also seen in the material which did not bind to concanavalin A.

The pH stability of the purified enzyme was tested by incubation overnight in 50 mM acetate or Tris-HCl buffers at 4 °C and then assaying with [^{3}H]cAMP at pH 7.5 as described under Experimental Procedures. The enzyme was found to be unstable below pH 6.0 yet tolerant of pH 6–10. This stability curve closely paralled the pH–activity curve in which the purified enzyme was assayed with 100 μM cAMP in an overlapping series of 50 mM buffers from pH 2 to 10. In two separate experiments, the enzyme was found to have maximal activity in Hepes at pH 7.5, with a decrease at both higher and lower pH. Isoelectric focusing was done for both the purified enzyme and the "salt peak" activity. The purified enzyme had a p*I* of 8.3 ± 0.1 (n = 5) while the salt peak activity precipitated in the gel and was equally distributed in the pH gradient.

Regulation of Culmination-Stage PDE by an Endogenous Effector. The extracellular form of PDE which functions during chemotactic migrations of individual *Dictyostelium* amoebae can be activated by preincubation of the enzyme with magnesium and DTT (Chassy, 1972; Kessin et al., 1979). This treatment is known to remove an endogenous protein from the extracellular PDE and thus lower the apparent K_m for cAMP from a millimolar to a micromolar range. We investigated the culmination stage enzyme for this mode of regulation as shown in Table II. Preincubation of purified culmination stage PDE with an excess of partially purified extracellular inhibitor from the aggregation stage resulted in nearly complete loss of activity. Activation of this inhibited preparation with Mg–DTT restored 90% of the original activity. Treatment with DTT alone restored 50% of the activity while $MgSO_4$ alone and KCl alone were without effect. If the inhibitor was treated with Mg–DTT prior to incubation with the enzyme, no inhibition occurred. Thus, the enzyme from the culmination stage can be regulated by the extracellular inhibitor from the aggregation stage. These results indicated

Table II: Inhibition and Activation of Culmination-Stage Phosphodiesterase[a]

preincubation	PDE activity without inhibitor	PDE activity with inhibitor
buffer control	9.3	0.4
0.1 M $MgSO_4$ + 5 mM DTT	9.2	8.0
5 mM DTT	9.4	4.5
0.1 M $MgSO_4$	10.0	0.5
1 M KCl[b]	10.0	0.7
inhibitor + Mg-DTT[c]	9.8	9.2
culmination 100000*g* supernatant (10 units) + purified PDE (4 units)[d]	14.6	
extracellular fluid from culmination stage[e] + purified PDE (10 units)	9.8	

[a] Purified PDE was preincubated 30 min with an excess of partially purified extracellular inhibitor (6.5 units of PDE plus 5 units of inhibitor in 0.7 mL). This preparation and PDE alone were incubated overnight as described in the table. The following day, the samples were assayed with 100 μM [^{3}H]cAMP as described under Experimental Procedures. Values are units per milliliter. The PDE reaction is inhibited 15–20% by the presence of the 0.5–1 mM DTT contributed by the treated enzyme. Tabulated activity has been corrected for this inhibition. [b] Effect of high salt concentration on PDE inhibitor binding. [c] Inhibitor was incubated overnight with Mg-DTT prior to a 30-min incubation with PDE. [d] Test for the presence of free inhibitor in culmination 100000*g* supernatant. [e] Prepared as described under Experimental Procedures.

that the enzyme from the culmination stage was subject to the same regulatory property as the enzyme which functions during chemotaxis. However, when the extracellular fluid from the culmination stage was collected and assayed for heat-released inhibitor, no inhibition was observed, while identical treatment of aggregating cells yielded high levels of inhibitor. In addition, mixtures of purified PDE and a 100000*g* supernatant gave additive values for PDE activity (Table II). Thus, a culmination stage inhibitor could not be demonstrated when extracted by the methods used to obtain the inhibitor from the aggregation stage.

Nature of Activation and the Apparent K_m of Phosphodiesterase. Activation of the extracellular PDE by magnesium and DTT was first reported by Chassy (1972). Recently, Kessin et al. (1979) have shown that the activation procedure removes an inhibitor from the extracellular enzyme and that the binding of the inhibitor inhibits the enzyme by converting its K_m from the micromolar to the millimolar range. Several micromolar kinetic constants have been reported for the *Dictyostelium* extracellular PDE (Chassy, 1972; Pannbacker & Bravard, 1972; Toorchen & Henderson, 1979). Toorchen & Henderson (1979) found three forms of PDE differing in molecular weight but with similar K_m values of 2–10 μM. We examined the kinetics of nonactivated 100000*g*-soluble PDE and the activated purified enzyme from the culmination stage for comparison to the extracellular forms from the aggregation stage (Figure 2). In the micromolar substrate range (0.75–148 μM cAMP), both the activated and the nonactivated culmination stage enzymes had an identical apparent K_m of 10 μM. At higher substrate concentrations (0.5–7.7 mM cAMP), however, the nonactivated preparation had an additional K_m of 2 mM. This high K_m activity is characteristic of inhibited PDE (Kessin et al., 1979). A K_m of 2 mM was also reported for the nonactivated extracellular enzyme (Chang, 1968). We found that the activated 100000*g*-soluble fraction had only the low K_m form as did the enzyme from all steps of the purification procedure.

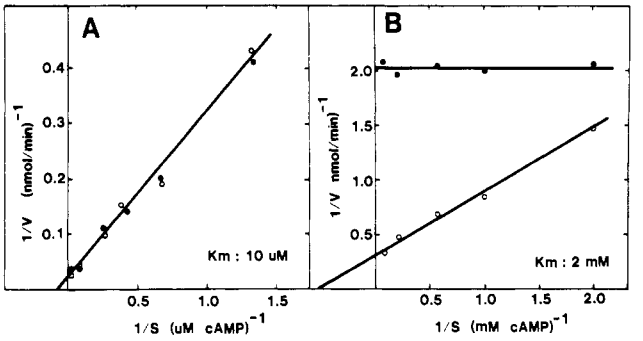

FIGURE 2: Kinetics of soluble phosphodiesterase before and after activation by Mg–DTT. Lineweaver–Burk double-reciprocal plot of the activity of purified PDE (●) and total 100000*g*-soluble, nonactivated PDE (○), both diluted to an activity of 30 units/mL (nanomoles of cAMP per minute) when assayed at 100 μM cAMP. The substrate range was 0.75–148 μM (A) and 0.5–7.7 mM cAMP (B). Activity was assayed with [^{3}H]cAMP as described under Experimental Procedures. Points are the average of triplicate determinations. The line was fitted by the method of least squares.

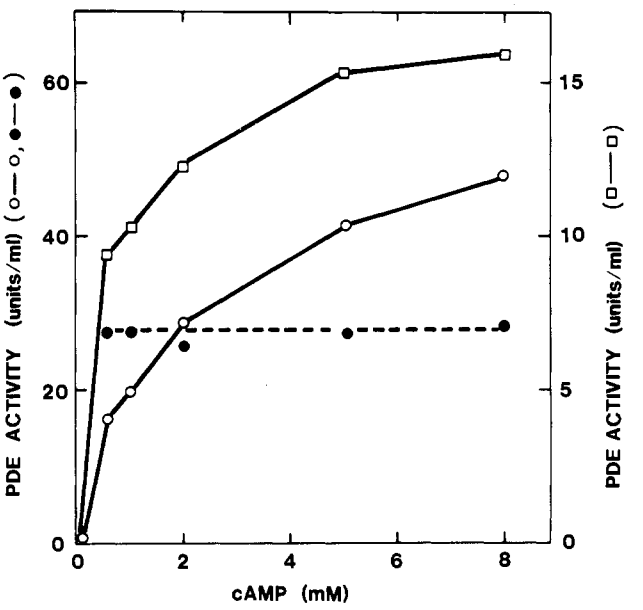

FIGURE 3: Reconstitution of high K_m phosphodiesterase: 35–75% ammonium sulfate fraction of a nonactivated soluble culmination-stage protein (○); same material which had been activated overnight with Mg–DTT, as described under Experimental Procedures (●); activated preparation incubated with 15 units/mL partially purified extracellular PDE inhibitor (to 60% inhibition when assayed at 100 μM cAMP) (□). Activity was measured with [^{3}H]cAMP as described under Experimental Procedures.

Proof that the 2 mM K_m was the result of inhibitor binding was obtained by reconstitution of the inhibited enzyme from the low K_m enzyme and a partially purified inhibitor (Figure 3). An enzyme preparation which had not been treated with Mg–DTT showed increased PDE activity with increasing substrate from 0.5 to 7.7 mM cAMP. The same material which had been activated with Mg–DTT, as described under Experimental Procedures, showed activity that was saturated at all substrate concentrations. The activated preparation was then incubated with 15 units/mL partially purified extracellular PDE inhibitor (to 60% inhibition when assayed at 100 μM cAMP). As shown in Figure 2, this treatment converted the enzyme back to the high K_m form.

Effect of Mg–DTT Treatment of the Enzyme on Its Behavior during Gel Filtration Chromatography. Although an endogenous inhibitor was not demonstrable in the extracellular fraction from the culmination stage, further fractionation of

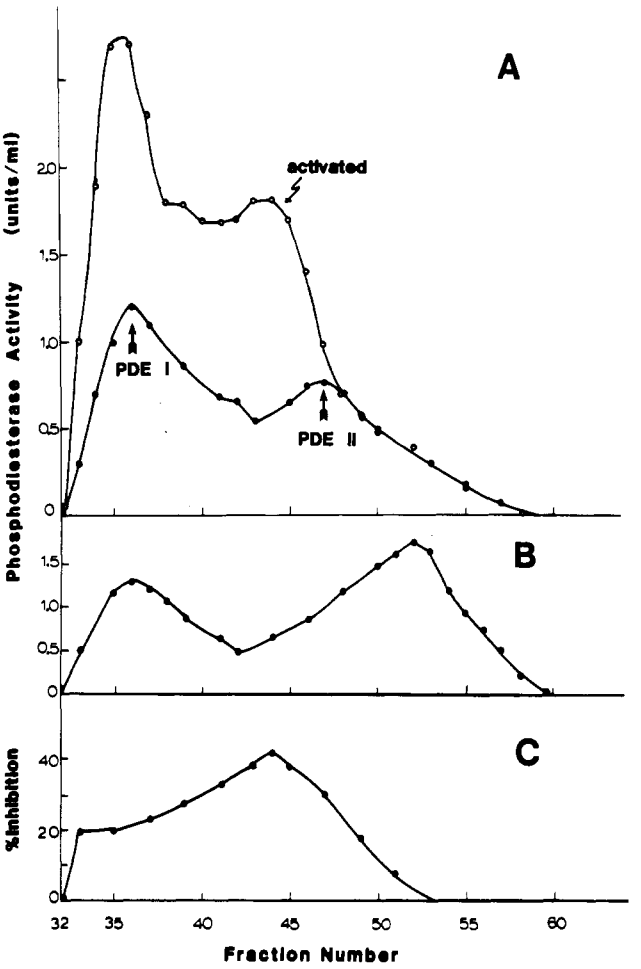

FIGURE 4: Composite profile of soluble culmination stage phosphodiesterase from a column of Bio-Gel P300. Samples (5 mL) were applied to the column (2.6 × 92 cm) and eluted with 100 mM Tris-HCl, pH 8.5, at 26 mL/h. (A) Elution of nonactivated soluble PDE (●); same fractions after overnight incubation in the Mg-DTT activation mixture (○). (B) Elution of activated soluble PDE. (C) Inhibition of purified PDE by heated fractions of (A). Fractions (3.5 mL) were assayed for PDE with 100 μM [^{3}H]cAMP as described under Experimental Procedures (A and B). Inhibitor (C) was determined in fractions after heating at 80 °C for 10 min. Twenty microliters of the heated fraction was incubated with 0.2 unit of purified PDE for 30 min at 25 °C prior to assay.

the cell-associated enzyme revealed its presence. Figure 4 shows the results of an experiment in which the total soluble protein (0–70% ammonium sulfate fraction of a 100000*g* supernatant) was eluted from a Bio-Gel P300 column and then the fractions were assayed with and without "activation" treatment. Two peaks of activity were seen in the nonactivated material, designated PDE I (M_r >260 000, above the linear range of the column) and PDE II (M_r 95 000–100 000). When the fractions were individually activated, the high and intermediate molecular weight fracions increased in activity, while the low molecular weight peak did not. When the soluble preparation was activated prior to chromatography, a M_r 68 000 peak appeared (Figure 4B). Two peaks of activity (at M_r 68 000 and 260 000) were also found when this activated 100000*g*-soluble preparation was subjected to lectin chromatography on Con A–Sepharose prior to gel filtration chromatography. We also found that the purified enzyme showed a single elution peak at M_r 65 000–68 000 while the salt peak which separated from the purified enzyme on DEAE-Sephadex chromatography corresponded to a molecular weight of 250 000. Thus, the enzyme exists in more than one form, and these forms can be separated during the purification procedure.

Although we had not been able to demonstrate inhibitor activity in extracellular fluid or crude soluble fractions from the culmination stage, the column elution (Figure 4A,B), together with the activation studies of the purified enzyme (Table II), led to testing of the fractions from the Bio-Gel column for the inhibitor. Figure 4C shows the inhibition of purified culmination stage PDE by samples of individual fractions which had been treated at 80 °C for 10 min. Inhibitor was present in all the fractions which contained enzyme that was activated by Mg–DTT treatment (Figure 4A). The peak of inhibitor activity corresponded to fractions containing proteins having a molecular weight of 140 000. This elution pattern was reproducible in three separate tests of culmination stage homogenates. The fractions which contained the inhibitor (Figure 4C) were pooled, concentrated by lyophilization, and applied to a Sephacryl S300 column (not shown). The resulting fractions were assayed for inhibition by preincubation with the purified PDE as described under Experimental Procedures. The inhibitor eluted as a single peak corresponding to a molecular weight of 30 000–40 000. The results from Figure 4 indicate that Mg–DTT treatment of the 95 000–100 000 molecular weight form of the enzyme (PDE II) separates the enzyme and the inhibitor with resulting molecular weights of 68 000 and 30–40 000, respectively. The results also show that an endogenous inhibitor of PDE is present at the culmination stage but that it can be demonstrated only after partial purification of the enzyme.

Interaction of Culmination-Stage Phosphodiesterase and Its Inhibitor. Further evidence for the presence of PDE inhibitor at the culmination stage came from a study of the inactivation of the enzyme in an 8000*g* supernatant fraction. The soluble activity decreased markedly after centrifugation (Figure 5), while activity in the pellet declined more slowly. After standing overnight at 4 °C, the supernatant fluid contained less than 30% of its original activity, whereas the pellet value was nearly unchanged. However, activation treatment with Mg–DTT could restore the soluble activity to the same level as that obtained with fresh preparations, even after prolonged storage at 4 °C (several days) or after being frozen (several weeks at −20 °C). Therefore, we reasoned that the inhibitor was present in the culmination stage extracts and was sequestered in excess in some cellular compartment. Upon homogenization of the tissue, the inhibitor would be released from this compartment and would rapidly bind to the enzyme. Yet as described previously, we could not demonstrate free inhibitor in tissue homogenates. Inhibitor could only be observed after the native high molecular weight fractions from the Bio-Gel chromatography were heated (Figure 4). However, inclusion of 0.1 M calcium in the homogenization buffer reduced soluble activity by at least 80% (Figure 5). This was unexpected since neither calcium nor magnesium affected the activity of the purified enzyme nor did they alter the binding of enzyme and inhibitor. In addition, incubation of the calcium-treated extract with Mg–DTT restored the activity to the level of the non-calcium-treated control (Table III). This activation of the calcium-treated enzyme and the time course of inactivation (Figure 5) suggested that the presence of calcium in the homogenizing medium somehow resulted in release of the PDE inhibitor. The reduction of PDE activity was found to occur only when Ca^{2+} was present during extraction, as subsequent addition of calcium to either supernatants or pellets had no effect on PDE activity. We also found that heat treatment of the Tris–calcium supernatants release inhibitor activity. Table III shows that both the calcium-containing 8000*g* supernatants and the calcium-con-

Table III: Effect of Calcium on PDE from the Culmination Stage of Development[a]

sample	PDE[b] activity		activated PDE[c]		inhibition by heated fraction[d]	
	Tris	Tris–Ca	Tris	Tris–Ca	Tris	Tris–Ca
crude homogenate	13.0	11.0	22.0	23.0		
8000*g* supernatant	5.0	2.0	12.0	13.0	0	53
8000*g* pellet[e]						
Tris supernatant	1.5	0.7	3.5	1.5	4	2
Tris pellet	7.0	7.5	12.0	11.0		
calcium supernatant	2.0	1.0	6.0	2.5	40	12
calcium pellet	6.5	8.0	11.0	11.5		

[a] Lyophilized culmination tissue was homogenized at 20 mg dry weight/mL in 25 mM Tris-HCl, pH 8.5 (Tris), or the same buffer containing 0.1 M $CaCl_2$ (Tris-Ca). [b] Phosphodiesterase activity was assayed with 100 μM [^{3}H]cAMP as described under Experimental Procedures. Activity is expressed as nanomoles per minute per milliliter. [c] After extraction with either Tris or Tris–Ca, the samples were activated with Mg–DTT as described under Experimental Procedures prior to assay. [d] The 8000*g*-soluble fractions were heated 10 min at 80 °C and then preincubated with purified PDE (20 mL/0.5 unit) for 30 min prior to assay. Inhibition is expressed as the percent of untreated control enzyme at the same dilution. [e] The 8000*g* pellet from each treatment was divided in half, homogenized again in Tris or Tris–Ca, and centrifuged again at 8000*g*, and all pellets were suspended in Tris. The supernatants of this second homogenization were tested for inhibitor.

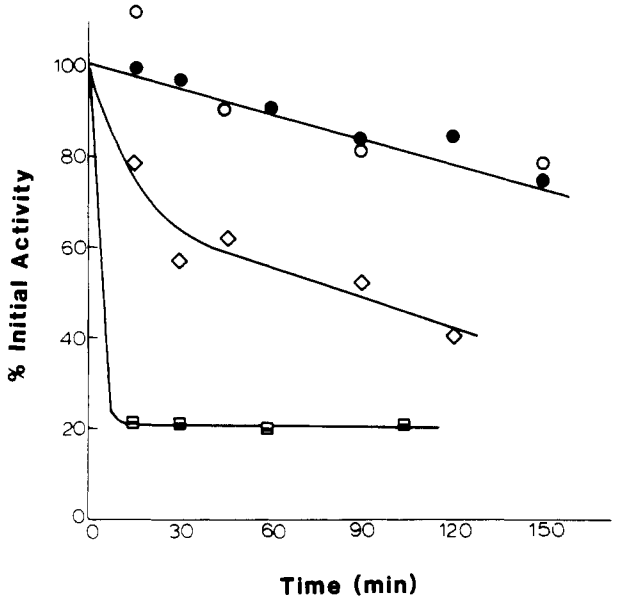

FIGURE 5: Time course of inactivation of PDE in homogenized culmination tissue. Lyophilized cells in the culmination stage were homogenized at 20 mg dry weight/mL in 25 mM Tris-HCl, pH 8.5, centrifuged for 30 min a 8000*g*, and assayed with 200 μM [^{3}H]cAMP at intervals. Tissue was kept in an ice–water bath during the experiment. (●) Crude tissue homogenate; (○) 8000*g* pellet resuspended in Tris-HCl; (◇) 8000*g* supernatant; (□) 8000*g* supernatant of culmination tissue homogenized in Tris-HCl containing 0.1 M $CaCl_2$. The calcium supernatant is plotted with reference to the initial activity of a Tris-HCl homogenate (without calcium) prepared at the same time.

taining washes of the pellets become inhibitory on heating, while Tris supernatants do not.

It remains unclear whether inhibitor alone or enzyme–inhibitor complexes were released from the pellet, although in a series of experiments on both aggregate and culmination tissue differential behavior of the enzyme and the inhibitor was observed. For example, activation treatment increased the 8000*g* pellet enzyme activity (at 100 μM cAMP) (1.6 ± 0.1)-fold (n = 14), whether the samples were homogenized in the presence or absence of calcium. Thus, the samples in Ca^{2+} showed large increases of inhibitor in the supernatants (see Table III) but no change in pellet activation. Also, an 8000*g* pellet from culmination tissue was rehomogenized in buffer containing a range of Ca^{2+} concentrations (5 μM–100 mM) and Lubrol PX detergent (0.2–1.0%). The calcium released only 10–25% of the pellet-bound PDE activity but large amounts of inhibitor, while the detergent released 50–60% of the pellet PDE units and no inhibitor. All tissue preparations released inhibitor when heated in the presence of calcium. The optimum calcium concentration for release of inhibitor from supernatants (when calcium was added after they were prepared but prior to heating) was in the range of 0.5–5 mM, while inhibitor release from pellets during homogenization required higher levels.

Since magnesium and calcium together are known to modulate cyclic nucleotide metabolism in many systems, a final test was made on the effect of Mg^{2+} concentration on the heat release of inhibitor from culmination-soluble protein. In three trials utilizing both $MgCl_2$ and $MgSO_4$, we found that, unlike calcium, millimolar concentrations of Mg^{2+} appeared to stabilize the enzyme–inhibitor complex and prevent heat release of inhibitor, whereas at micromolar levels considerable inhibitor was released. When both Mg^{2+} and Ca^{2+} were present together at millimolar concentrations, inhibitor was released to the same extent as if Ca^{2+} alone were present.

Inhibition of PDE by Anti-PDE IgG. Antibodies to the purified PDE were obtained from serum as described under Experimental Procedures and were used to investigate the relationship of the activity found at various steps of the purification, the activity at different stages of development, and the activity in the presence of the endogenous inhibitor (Table IV). Anti-PDE IgG inhibited the activity of the purified enzyme up to 70–80%, whereas preimmune serum was not inhibitory. Other soluble forms of PDE were also inhibited by antiserum to the purified enzyme, including the salt peak from the DEAE-Sephadex chromatography. The two molecular weight peaks (PDE I and PDE II) which can be separated by gel filtration from untreated culmination-soluble protein were also inhibited (not shown). These results suggested that the M_r 68 000 PDE was a common component of the various forms of the enzyme. Since Mg–DTT treatment appeared to convert much of the high molecular weight activity into the smaller form by removing inhibitor (Figure 4), enzyme–inhibitor complexes (E·I) were reconstituted from purified components and tested for inhibition by the anti-PDE IgG. Table IV shows that for E·I at half-maximal inhibition ($I_{0.5}$) the anticatalytic activity of the antibody was as efficient as for the inhibitor-free enzyme. At E·I close to inhibitor saturation (I_{max}), the antibody effect was reduced but not eliminated. Thus, the anti-PDE IgG continued to "recognize" the enzyme after it was bound to the extracellular inhibitor. The antiserum against the purified culmination stage enzyme was also tested against crude (not activated) extracts from the aggregation, pseudoplasmodium, and culmination stages. The activity from all three stages could be inhibited, indicating that

Table IV: Inhibition of Phosphodiesterase by Anti-PDE IgG[a]

amount of IgG (μL)	inhibition[b] purified PDE	salt peak	E·I, $I_{0.5}$	E·I, I_{max}	aggregation	slug	culmination
2.2	34	40	54	14	5	0	10
4.4	61	65	62	23	0	10	0
9.3	67	70	65	36	0	0	4

[a] Purified phosphodiesterase (105 units/mL) was preincubated 1 h at 25 °C with Tris-HCl buffer, purified inhibitor at half-saturation (E·I, $I_{0.5}$), and a saturating concentration of inhibitor (E·I, I_{max}). Samples (10 μL) of each were mixed with IgG as indicated, held overnight at 4 °C, and assayed with 100 μM [^{3}H]cAMP as described under Experimental Procedures. Samples of salt peak enzyme (46 units/mL) and 20 mg dry weight/mL homogenates of mass cultures in the aggregation, slug, and culmination stages were treated with antibody in the same way. Crude homogenates contained 8–12 units/mL nonactivated PDE. [b] Activity is expressed as the percent inhibition of controls incubated without IgG.

the protein is the same at all stages of differentiation or at least shares the same antigenic sites.

Discussion

The extracellular PDE (ePDE) from aggregating amoebae has been found to exist in several forms, from both untreated supernatants (Chang, 1968; Pannbacker & Bravard, 1972), activated supernatants (Chassy, 1972; Toorchen & Henderson, 1979), and supernatants of inhibitor-deficient mutants (Dicou & Brachet, 1979). The activated or inhibitor-free enzyme is a low molecular weight form (M_r 65 000) with a high isoelectric point (pI 8.3). This undoubtedly corresponds to the purified cell-associated enzyme from the culmination stage which is described in this paper (Figure 4). The results of this work also show that the properties of the culmination PDE are dependent on its stage of purification, which may reflect loss of molecules that are normally associated with PDE in vivo. For example, the anti-PDE IgG inhibits the activity in dissected tissue sections but not crude homogenates. All soluble forms of the enzyme show specific elution from Con A–Sepharose, but crude soluble supernatants do not. Mammalian phosphodiesterases are known to be associated with other proteins including calmodulin, cAMP-dependent protein kinase (Donnelly, 1978), and a specific macromolecular inhibitor (Wallace et al., 1978).

The results described here demonstrate the presence of a PDE inhibitor from cell homogenates of the culmination stage, in both the soluble and pellet fractions. It appears to have a similar molecular weight (30 000–40 000) and mechanism of inactivation by alternation of the K_m as the extracellular inhibitor of PDE from the aggregation stage (Frank & Kessin, 1981). However, the inhibitor from the culmination stage cannot be demonstrated without addition of calcium or partial purification of the enzyme.

The fact that calcium influences PDE activity in culmination-stage homogenates may be significant in view of the recent expansion of information concerning calmodulin and cyclic nucleotide regulatory systems. Calmodulin, which is ubiquitous in eukaryotes and highly conserved throughout evolution (Cheung, 1980), has been isolated from *Dictyostelium* and localized intracellularly with a fluorescent-conjugated antibody (Clark et al., 1980). It activated the bovine brain calcium-dependent PDE but was not found to affect *Dictyostelium* PDE. We also did not find any Ca^{2+} stimulation of PDE activity at any stage of purification. Although ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) treatment caused large losses of soluble activity, suggesting calcium regulation, the effect was not reversible by calcium. A gel filtration profile of PDE from culmination supernatants also did not indicate the loss of a particular form of the enzyme in the presence of EGTA. In view of the results reported here, the inability of calcium to alter the activity of the purified activated enzyme is probably not unexpected. We believe that the purified PDE represents a core activity or catalytic subunit which is no longer subject to modulation (except, apparently, inhibitor binding) as has been found in other systems (Klee et al., 1980; Epstein et al., 1978; Thompson et al., 1979).

This research provides evidence, however, that calcium is involved in regulation of PDE activity. Heating crude soluble extracts in the presence of 1–5 mM Ca^{2+} released inhibitor activity (Table III). Homogenization in 0.1 M Ca^{2+} released inhibitor, but not PDE, from the particulate fraction. Although the calcium levels required were much higher than the micromolar concentrations which saturate calmodulin binding (Klee et al., 1980), this does not preclude a calmodulin involvement. Transient calcium fluxes into *Dictyostelium* cells are known to occur upon cAMP stimulation (Wick et al., 1978). In addition, calcium has been shown to be intensely localized in the prestalk and stalk cells and to induce stalklike differentiation in cells plated on high extracellular concentrations (100–150 mM) (Maeda & Maeda, 1973). Aggregation is also affected by micromolar variations in extracellular calcium. Thus, both systems in which cyclic nucleotide PDE is believed to be an important component, signalling (Malchow et al., 1975) and stalk development (Maeda & Maeda, 1973), are also circumstantially connected with increased calcium levels.

Purification and characterization of PDE from the culmination stage have provided some insight into the regulation of its activity during cellular differentiation. It appears that the enzyme is regulated similarly at the aggregation stage, where it acts to degrade the chemotactic signal, and at the culmination stage, where it is cell associated and is active in only one of the two cell types. It remains to be determined if the PDE activity is masked in one cell type by the inhibitor, if Ca^{2+} levels (and perhaps calmodulin) are involved in its regulation, and whether the biological function of the enzyme remains that of modulating a chemotactic signal for cellular movements within the developing individual or of regulating intracellular levels of cAMP.

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Registry No. cAMP, 60-92-4; cAMP phosphodiesterase, 9036-21-9.

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Structure of Native Polysaccharide Antigens of Type Ia and Type Ib Group B *Streptococcus*†

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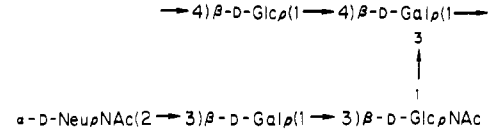
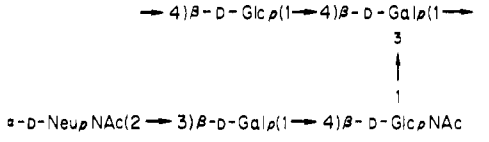
ABSTRACT: The native polysaccharide antigens isolated from the types Ia and Ib group B *Streptococcus* both contain D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and sialic acid in the molar ratio of 2:1:1:1. The structure of the native Ib antigen has been elucidated and is composed of the following repeating unit in which all the side-chain β-D-galactopyranosyl residues are masked by terminal sialic acid residues:



The structure of the native Ia antigen has also been elucidated and is composed of the following repeating unit:



The two native antigens are isomeric, differing only in the linkage of the side-chain β-D-galactopyranosyl residues to their respective 2-acetamido-2-deoxy-β-D-glucopyranosyl residues.

In early immunological studies Lancefield characterized the type-specific antigens (Ia, Ib, II, and III) of group B *Streptococcus* (Lancefield, 1933, 1934, 1938). An additional serotype (Ic) can also be defined on the basis of a type-specific protein antigen (Wilkinson & Eagon, 1971). The type-specific polysaccharide antigens were isolated by hot hydrochloric acid extraction of the streptococcal organisms (Lancefield, 1934, 1938) and thus resulted in structurally incomplete antigens. While still retaining type specificity, these acid-extracted antigens are immunologically incomplete and form a lower molecular weight core to their respective complete native antigens, which all contain additional terminal sialic acid

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