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CHARACTERIZATION OF MULTIPLE EXTRACELLULAR cAMP-PHOSPHODIESTERASE FORMS IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

The growth phase extracellular adenosine 3':5'-monophosphate phosphodiesterases from *Dictyostelium discoideum* have been studied. Following enzyme activation with dithiothreitol and magnesium chloride (1), three peaks of activity are resolved on DEAE-cellulose chromatography. The three forms are distinct with respect to: 1) mobility on non-denaturing polyacrylamide gel electrophoresis; 2) molecular weight; 3) sensitivity to an inhibitor protein.

The Michaelis constants (K_m) for cAMP of all three forms are similar and in the 1-10 μM concentration range: no evidence is found for an enzyme species with a K_m in the 1-10 mM concentration range or for monomer-dimer interconversion as noted by other workers (2).

INTRODUCTION

When deprived of a food source the initially free-living amoebae of the slime mold *Dictyostelium discoideum* commence a developmental process leading to chemotactic aggregation into multicellular masses and morphogenesis of the tissue-like aggregates to yield a fruiting body composed of a spore-containing head supported on a cellular stalk. The chemotactic agent is cAMP* (3) which is also thought to induce early biochemical events of differentiation (4,5) and possibly to participate in establishing the pattern for terminal stalk or spore cell differentiation (6,7,8). *D. discoideum* has elaborated a complex system of cyclic nucleotide phosphohydrolases (cyclic adenosine 3':5'-monophosphate 5'-nucleotidohydrolase, EC 3.1.4.17) to control cAMP concentrations during all phases of the life cycle (9).

While growing on bacteria or axenic broth (vegetative phase) the amoebae secrete phosphodiesterase (ePD) activity into the extracellular environment (10,11,12). The ePD activity is sharply inhibited at the late vegetative and early starvation stages by production of a heat stable inhibitor protein (13,14). A membrane-bound phosphodiesterase (mPD) activity appears and increases 10-15 fold during development, reaching a peak 8-11 hours after removal of the food supply (15, plus our unpublished observations). The ePD activity also increases during the developmental stage though not as dramatically as mPD (16).

*Abbreviations - cAMP, cyclic adenosine 3':5'-monophosphate; DTT, dithiothreitol; ePD, extracellular phosphodiesterase; mPD, membrane-bound phosphodiesterase; Tris, tris(hydroxymethyl)aminomethane.

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One of the major unresolved questions in this system involves the relationship between ePD and mPD. It is not known if one gene product is modified to produce these two forms or whether distinct or related genes are responsible. The issue is complicated by observations of heterogeneity in ePD. Previous workers (10,17) have described high and low K_m forms of vegetative ePD. Brachet *et al.* (18) have reported an inhibitor-resistant form of ePD present during development. This latter observation raises the additional question of whether there is any relationship between ePD of vegetative and developmental stages.

As a first step towards resolution of these questions, we have undertaken analysis of the vegetative ePD activity. We report here that the ePD activity is due to the presence of heterogeneous species.

EXPERIMENTAL METHODS

Strains and Growth Conditions. All experiments employed strain Ax-3 amoebae grown on autoclaved suspensions of *Escherichia Coli* B/r as described by Krichevsky and Love (19). Growth supernatant was harvested in late exponential or early stationary phase (6×10^6 - 1×10^7 cells/ml) by removal of cells at 500xg for 15 minutes.

Assay of cAMP Phosphodiesterase. The assay employed is a modification of the one described by Bergmeyer (20). Assays were performed at 30°C in a total volume of one ml containing 50 mM Tris-HCl (pH 7.5 at 30°C), 50 µM cAMP, 10 mM $MgCl_2$, 0.21 units alkaline phosphatase and 0.24 units adenosine deaminase. Absorbance at 265 nm was monitored continuously in a Beckman Model 25 thermostated spectrophotometer versus a water blank. Units are expressed as nanomoles 5'-AMP formed/min under these conditions. For Lineweaver-Burk analysis cAMP concentration was varied from 5 to 100 µM.

Purification of Vegetative ePD. The procedure of Chassy and Porter (1) was utilized without modification up to the DEAE-cellulose chromatography step. At this point the procedure was modified by using a smaller column (1.9 cm x 21 cm) and a total gradient volume equal to 500 ml. The limits of the gradient were identical to those in the procedure cited. Fractions of 5 ml were collected and assayed as described above.

Preparation of Inhibitor Protein. Inhibitor was prepared by heat treatment (10 minutes at 80°C) (15) of the dialyzed 90% $(NH_4)_2SO_4$ precipitate obtained from growth supernatant (Table I, step 2). Any precipitate which formed subsequent to heat-treatment was removed by centrifugation at 25,000xg for 30 minutes.

Assay of Inhibitor Protein. The ePD and inhibitor samples were preincubated in 300 µl containing 50 mM Tris-HCl (pH 7.5 at 30°C) and 10 mM $MgCl_2$ for 10 minutes at 30°C. The conditions described for ePD assay were then fulfilled and the reaction rate recorded.

Polyacrylamide Gel Electrophoresis. Electrophoresis on 5% polyacrylamide gels was performed as described by Davis (21). Activity staining for phosphodiesterase was performed as described by Goren *et al* (22). Use of a slab gel configuration enhanced the sensitivity of this technique. Gels were stained for protein using the perchloric acid-Coomassie G-250 stain described by Reisner *et al.* (23).

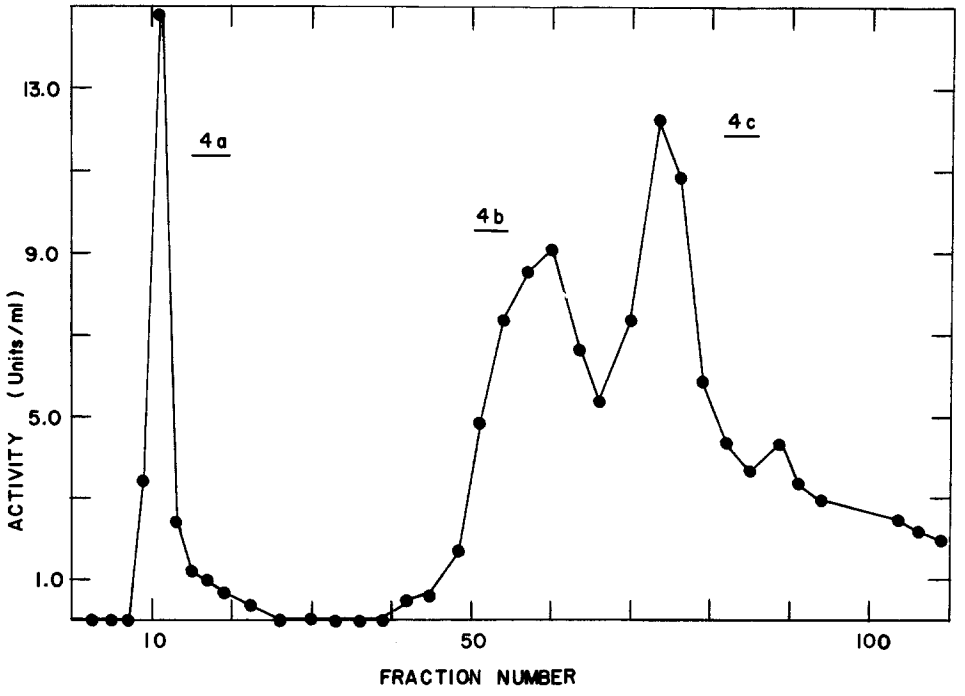

Figure 1: Chromatography of DTT-activated ePD on DEAE-cellulose. The procedure was as described in Experimental Methods section. Salt gradient elution was started at fraction #30.

Gel Filtration. A column of Sephacryl-200 (1.6 cm x 70 cm) was equilibrated with 0.1 M Tris-HCl (pH 7.5 at 5°C), 0.2 mM DTT. It was calibrated in four separate runs under identical conditions using blue dextran and the following proteins run two at a time: BSA (M.W. 6.6×10^4); RNAase A (M.W. 1.7×10^4); ovalbumin (M.W. 4.3×10^4); α-chymotrypsinogen A (M.W. 2.5×10^4); aldolase (M.W. 1.58×10^5).

Protein Determination. Protein was determined by the method of Bradford (24) (using Bio-Rad protein reagent and γ-globulin fractions as standard) or spectroscopically by the method of Christian and Warburg (25).

MATERIALS

cAMP, blue dextran 2000, alkaline phosphatase, adenosine deaminase, bovine serum albumin, ovalbumin, DTT, aldolase, α-chymotrypsinogen A, Tris, and Coomassie G-250 were all from Sigma Chemical Co., St. Louis, Mo. *E. coli* B/r were purchased as a washed, frozen cell paste from General Grain Processing, Muscatine, Iowa. DE-52 was from Whatman Ltd. Sephacryl-200 was from Pharmacia. Enzyme grade $(NH_4)_2SO_4$ was supplied by Schwarz-Mann. Glycine, acrylamide, protein assay reagent, and bis-acrylamide were from Bio-Rad. Bromophenol blue and ammonium persulfate were from Ames. All other chemicals used were reagent grade or better.

RESULTS

Fractionation of ePD. Little or no phosphodiesterase activity is detected in growth supernatants from early stationary phase cells or ammonium sulfate

TABLE I: Fractionation of ePD

Treatment	Vol (ml)	Units/ml	Protein mg/ml	Specific Activity	Yield %	Fold Purification
Growth supernatant	860	0.67	0.45	1.5	100	1
1st $(NH_4)_2SO_4$ fraction	61	2.5	2.3	1.1	27	1
Mg-DTT activation: 2nd $(NH_4)_2SO_4$ fraction	58	24	1.7	14	243	9.3
DEAE 4a	5	80	0.62	129	68	86
4b	5	97	6.0	16	86	11
4c	5	90	9.0	10	69	6.7

precipitates of these supernatants. Chassy (2) reported activation of the enzyme with exposure to DTT and $MgCl_2$; in our hands this procedure gives a 5-20 fold enhancement of activity. A probable basis for this activation is considered in the Discussion section. The activated ePD fractionates into several well-defined peaks on DEAE-cellulose chromatography (Figure 1). The first ePD peak emerges in the flow-through of the initial low-ionic strength wash. It is highly reproducible and corresponds to the fraction denoted 4a by Chassy (1). During salt gradient elution two other ePD peaks emerge and are denoted 4b and 4c. A typical fractionation scheme is shown in Table I. This procedure results in a partial purification of all three ePD fractions with 4a being the most highly purified. To study the relationship between these species we have characterized a number of properties as described below.

Electrophoretic Mobility. Enzyme activity can be localized on polyacrylamide gels using the activity stain of Goren *et al.* (22). Fraction 4a shows a single activity band which always co-migrates with a band comprising at least 90% of the input protein. Fraction 4b always has several closely spaced bands of enzyme activity. Fraction 4c gives only one activity band.

The electrophoretic mobilities of the ePD species detected by the activity stain are shown in Table II and are quite distinct, ranging from <0.1 to 0.58. The closely spaced activity bands of fraction 4b range in mobility from 0.1 to 0.3.

It has been reported that a 10 μM K_m ePD species of 65,000 daltons can dimerize by sulfhydryl oxidation to generate a species with a 2 mM K_m (2). The dimerization was reversed by dithiothreitol. We investigated this as a possible origin of our fractions.

TABLE II: Summary of Data

Fraction	Molecular Weight	K_m for cAMP	Electrophoretic Mobility
4a	65,000	6 μM	0.1
4b	95,000	10 μM	0.2
4c	185,000	2 μM	0.6

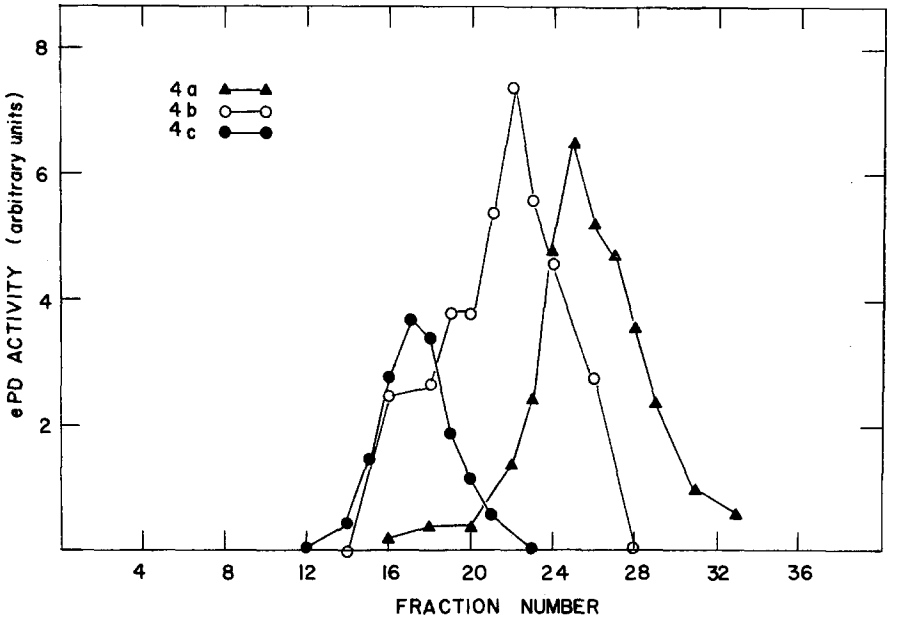

Figure 2: Chromatography of DEAE-cellulose ePD fractions on Sephacryl-200. A 1.5 ml sample of each ePD fraction was chromatographed separately (the figure is a composite elution profile) at a flow rate of 0.4 ml/min. Fractions of 3.0 ml were collected and assayed for ePD activity.

Molecular Weight Estimation. Molecular weight estimations were obtained by gel filtration (Figure 2) on a Sephacryl-200 column calibrated as described in Experimental Methods. The values obtained were 65,000, 95,000 and 185,000 for 4a, 4b and 4c, respectively, as summarized in Table II. Comparison of the peak widths of the ePD species with those of the calibration proteins indicates that with respect to size 4a and 4c represent homogeneous ePD forms while 4b may be a mixture of forms, one of which co-migrates with 4c. While the 65,000 species corresponds to the monomer reported by others, the molecular weights determined for 4b and 4c are not explained by dimerization of 4a.

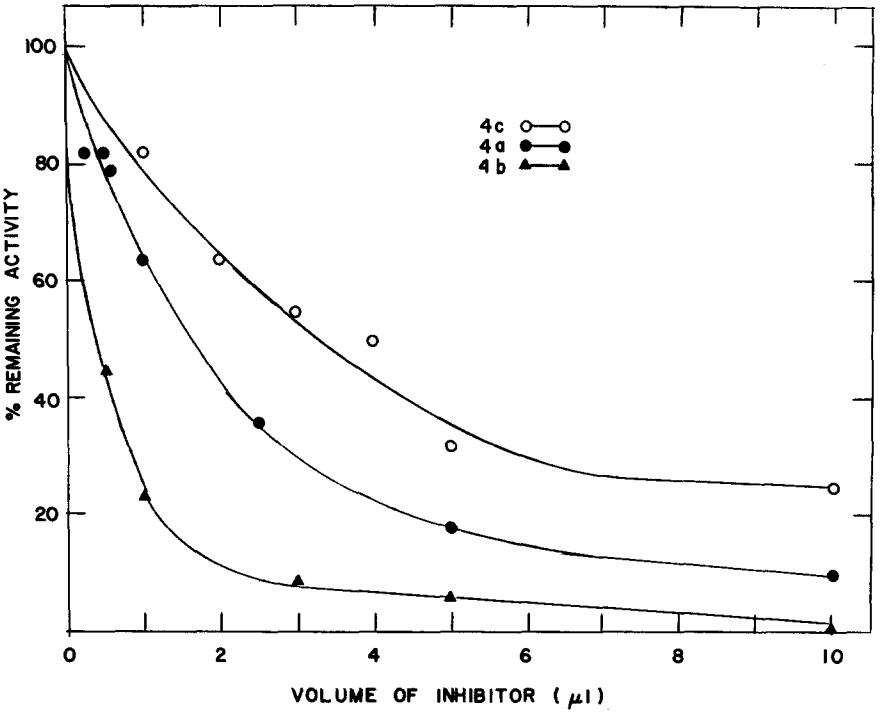

Figure 3: Sensitivity of ePD fractions to inhibitor. A 0.5 unit sample of each ePD fraction was preincubated with the indicated amount of inhibitor as described in Experimental Methods. Assay conditions were completed and the reaction rate recorded.

Kinetics. Previous workers have reported partial purification of ePD species with K_m values for cAMP of 2 mM (10) and 15 μM (2, 17). All the ePD fractions from DEAE-cellulose chromatography have K_m's for cAMP in the 1-10 μM range. The actual values obtained by double-reciprocal analysis of initial rate data are given in Table II. Within the accuracy limits of the experiment these K_m values are essentially identical.

Interaction with Inhibitor Protein. Riedel and Gerisch (12) established that *D. discoideum* produces a heat stable, 40,000 dalton (9) protein inhibitor of ePD in the early starvation phase of the developmental program. Under the growth conditions utilized for this study the inhibitor is present at late exponential phase. Heat treatment of the dialyzed 90% $(NH_4)_2SO_4$ precipitate from growth supernatant (Table I, step 2) releases active inhibitor. Titration of equal input units of the ePD fractions with inhibitor generates the response curves shown in Figure 3. Fraction 4b is most sharply inhibited while 4c is least susceptible. Fraction 4a is intermediate between 4b and 4c in its sensitivity to the inhibitor protein.

Interestingly, active inhibitor cannot be recovered from any fraction subsequent to DTT-Mg^{+2} activation (Table I, step 3). If the inhibitor preparation is made 5mM in DTT and incubated for 1 hour at 22°C, it fails to inhibit ePD activity while control inhibitor is still fully active. It thus appears that the DTT-Mg^{+2} activation step routinely used in enzyme fractionation causes irreversible inactivation of the inhibitor.

DISCUSSION

The vegetative ePD from *D. discoideum* can be fractionated into three distinct forms by DTT activation and DEAE-cellulose chromatography. These species differ in a number of properties. Their apparent molecular weights are 65,000, 95,000, and 185,000. Other workers have reported that the lowest molecular weight form can undergo dimerization with an associated change to a higher Michaelis constant. We do not observe this 130,000 dalton dimer nor do we observe a high K_m form.

The three forms we observe also have distinct electrophoretic mobilities on non-denaturing polyacrylamide gels. The fact that the fastest migrating electrophoretic form has the highest molecular weight and the slowest migrating form has the lowest molecular weight indicates that these species have widely differing charge-to-mass ratios. A model which invokes simple monomer-dimer interconversion is not adequate to account for the heterogeneity observed; however, some form of self-association of ePD cannot be completely ruled out. This would require a significant change in Stokes radius to explain the molecular weight data and a considerable change in charge-to-mass ratio to be consistent with the electrophoretic mobilities.

A number of alternate hypotheses which are reconcilable with our data can be considered. First, we have shown that treatments with dithiothreitol which activate the enzyme cause irreversible inactivation of the inhibitor. It is not known whether these treatments completely release ePD and inhibitor from their complexed form. It is possible that the ePD forms represent degrees of complexation of ePD with bound but inactivated inhibitor. All species reported here are inhibitor sensitive but the stoichiometry of inhibitor-to-enzyme in the complex(es) is not known.

The ePD forms could also represent products of distinct genes with inherently different physical but similar kinetic properties. If so, the genes may be related since the polypeptide inhibitor is effective against all forms.

Post-translational modifications of the same gene product could also account for the generation of differing charge-to-mass ratio by phosphorylation, glycosylation or proteolysis.

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