CHARACTERIZATION AND LOCALIZATION OF A CYCLIC AMP DEPENDANT PROTEIN KINASE FROM DICTYOSTELIUM DISCOIDEUM

by

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(ABSTRACT)

A developmentally regulated cyclic AMP-dependent protein kinase has been recently reported in Dictyostelium discoideum. This report describes some of the physical and kinetic properties of the cAMP dependent holoenzyme and its subunits. Gel filtration data suggests a holoenzyme Mr of 170,000-190,000, and catalytic and regulatory subunit Mrs of 40,000 and 49,000, respectively. These molecular weight determinations are compatible with an R_2C_2 subunit arrangement of the holoenzyme. Kinase activity required the presence of Mg^{2+} but cAMP binding to the enzyme was not dependent on divalent metal ions. The pH optimum for kinase activity was 7.5; the cAMP binding activity was not affected over a pH range of 5.0-10.0. The holoenzyme and isolated regulatory subunit had identical cAMP Kds of 28 nM.

Cyclic AMP was able to dissociate the subunits when analyzed by density gradient centrifugation. Histone VII-S activated the subunits in the absence of cAMP but did not produce their dissociation. In contrast to the gel

filtration data, sedimentation values indicated a dimeric holoenzyme structure. Reassociation of the subunits in the absence of cAMP occurred rapidly and was not dependent upon a preincubation with MgATP. High NaCl and low pH depressed both the total kinase activity and the ability of the subunits to reassociate as determined by activity ratio. MgATP did not decrease the ability of the holoenzyme to bind cAMP, neither did the holoenzyme possess a high affinity MgATP binding site.

By the use of microdissection techniques holoenzyme levels were determined in individuals at each stage of development and in each cell type during development. Kinase activity was low and non-cAMP dependent in early aggregates but increased and became cAMP-dependent in later aggregates. Maximum activity and cAMP-dependency occurred during the slug and culmination stages. The only differential distribution of the kinase within a single stage occurred during culmination when the activity in the stalks was approximately one-fourth that in the prespore mass. Preliminary evidence indicates that this difference is not due to an inhibitor.

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Chapter 1 INTRODUCTION

1.1 *Dictyostelium discoideum as a model system for development*.

The cellular slime mold *Dictyostelium discoideum* has been used with ever-increasing popularity as a simple model system with which to study a variety of developmental processes. (reviewed by Loomis, 1982). This organism can exist indefinitely in a unicellular state under conditions of adequate nutrient supply. Upon starvation, however, the cells in the population undergo a process of aggregation resulting in the formation of a multicellular organism typically consisting of 10,000-100,000 cells. During the remainder of the twenty-four hour developmental cycle the cells within each aggregate undergo simultaneous processes of morphogenesis and differentiation resulting in the formation of two cell types. After the completion of aggregation the cell mass elongates, becomes enclosed by a slime sheath and commences a variable period of migration (Raper 1940). In the absence of disruption the cells in the anterior third of the pseudoplasmodium, or slug, are destined to become stalk cells, while the cells in the posterior two-thirds of the slug will become spore cells

(Raper 1940, Bonner 1952). When migration ceases the anterior cells become situated atop the center of the cell mass and the presumptive stalk cells begin to migrate downward through the center of the prespore mass. At this time the prestalk cells begin to form thick cells walls and a cellulose sheath which confer rigidity to the stalk region, and allow the prespore mass to be lifted off the substratum (Bonner 1971). Cells from the prestalk region continue to migrate towards and through the central region (the papilla or tip), thereby increasing the height of the stalk in a process termed culmination. The cells within the stalk sheath eventually become highly vacuolated and die (Raper and Fennell 1952) while the spore cells become enclosed in a spore case (Hohl and Hamamoto 1969), and remain viable. When conditions are favorable the spores germinate and re-initiate the cycle. A diagrammatic representation of the life cycle is shown in Figure 1. This organism thus lends itself well to the study of processes which control differentiation for several reasons. The cells are genetically identical, the growth phase is distinct from the developmental phase, only two types of cells are formed, and the cells are separated into a simple pattern.

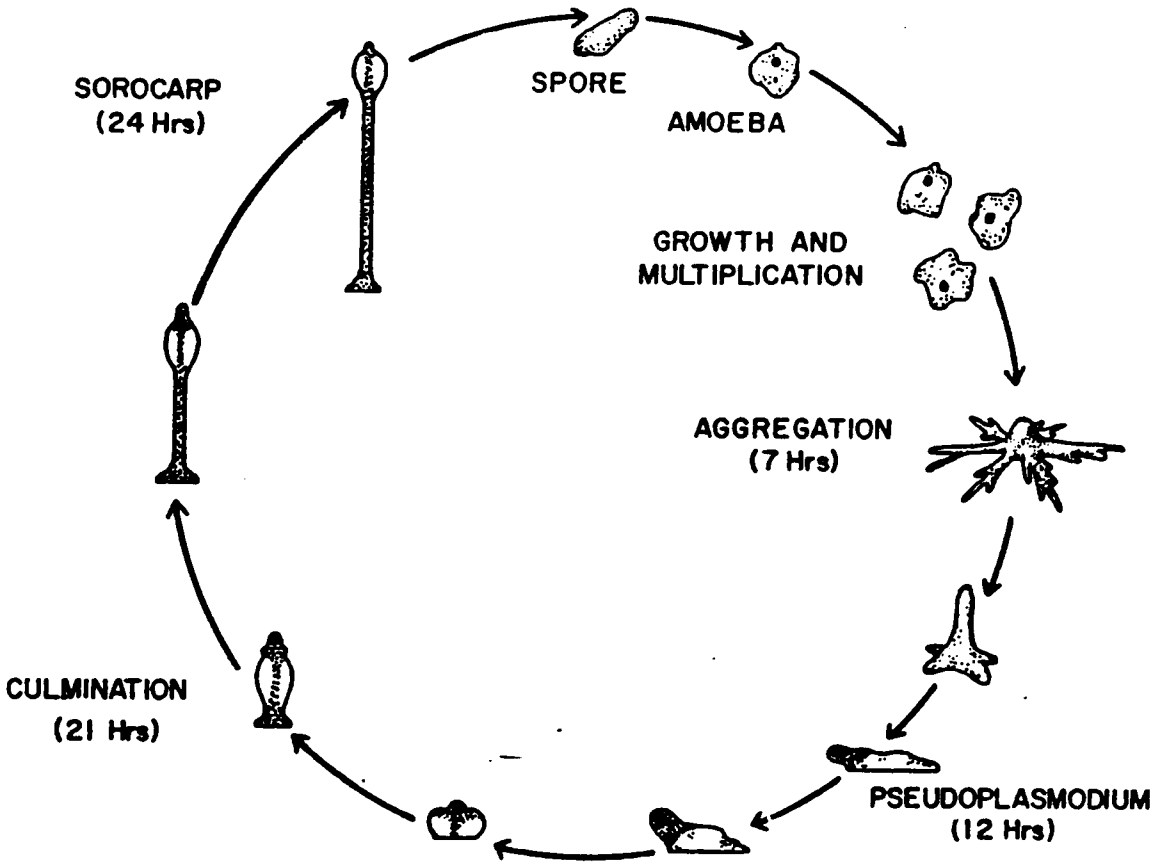


Fig.1. THE DEVELOPMENTAL CYCLE OF
Dictyostelium discoideum

1.2 The role of cAMP in development in Dictyostelium.

The agent responsible for inducing chemotaxis early in development is adenosine 3'5' cyclic monophosphate (cAMP) (Konijn et al. 1968). A complicated and highly regulated system exists for relaying the cAMP signal throughout the population of amoebae during aggregation. This system has been studied in considerable detail (see Loomis 1982). Interestingly, cAMP also appears to be involved in the later events of development. This has been shown by a variety of methods using both wild-type and mutant cells. Many of these effects have been determined by using methods which separate the normal processes of morphogenesis and differentiation. This is done by shaking aggregation-competent cells in solution which prevents normal development by preventing cell contact or by disaggregating slug-stage cells and shaking them to prevent re-entry into aggregation (Bonner 1970).

In experiments of this type it has been found that cAMP is required for cells to undergo differentiation. For example, a small percentage of wild-type cells treated with cAMP will undergo differentiation into clumps of stalk-like cells (Bonner 1970), while the mutant strain P-4 undergoes up to 100% stalk cell differentiation when exposed to cAMP (Chia 1975). Gross et al. (1981) found that cAMP was

required for stalk cell differentiation in the wild-type variant V12 and its sporogenous derivatives under certain conditions. Cyclic AMP also appears to be related to spore cell differentiation. When cells of whole pseudoplasmodia were subjected to a localized source of cAMP, two thirds of the cells adjacent to the source differentiated into stalk cells while one-third of the cells became spore cells (Feit et al. 1978). It was also found that cAMP potentiates prespore and spore cell differentiation in monolayer-grown cells of V12M2 and its sporogenous derivatives (Kay 1982, Town et al. 1976, Kay et al. 1978). Finally, in an experiment of the converse process it was seen that cAMP inhibited the rate of dedifferentiation of disaggregated cells (Finney et al. 1980).

The mechanism by which cAMP induces cells to differentiate is far from clear. However, several lines of evidence implicate cAMP in transcription and translation of late-stage genes. When starved cells in shaking culture are treated with high levels of exogenous cAMP several developmentally regulated proteins are induced prematurely (Gerisch et al. 1975, Darmon et al. 1975, Town and Gross 1978, Takemoto et al. 1978), although the expression of at least one protein (discoidin 1) is inhibited by cAMP (Williams et al. 1980). In addition many proteins that

would normally disappear upon disaggregation are maintained in the presence of cAMP (Landfear and Lodish 1980). Finally, cAMP stimulates the synthesis of most late-development mRNAs and may also slow the rate of their degradation during disaggregation (Chung et al 1981, Landfear et al 1981, Blumberg et al. 1982).

In addition to a possible role in inducing cell differentiation, cAMP may also be involved in regulating the prestalk-prespore pattern. For instance, when front and rear sections of slugs are separated each will normally undergo a reapportionment of cell types to produce a smaller but normally proportioned slug (Raper 1940, Bonner and Slifkin 1949). However, if the halves are grown in the presence of high cAMP levels, the prespore sections degenerate while the prestalk halves complete development (George 1977). Other experiments show that the tip region, which may contain high levels of cAMP (Pan et al. 1974, Brenner 1977), can induce reorganization and re-differentiation of slug cells when grafted onto the sides of pseudoplasmodia (Rubin and Robertson 1975). Finally, several studies show that prestalk cells are chemotactic towards or more responsive than prespore cells to sources of cAMP (Maeda and Maeda 1974, Sternfeld and David 1981, Matsukuma and Durston 1979). It has been postulated that

this may be a mechanism for the morphogenetic movements of cells during slug formation or culmination by inducing prestalk cells to move toward the tip region.

Because of the possibility that cAMP may regulate cell-type differentiation or pattern formation, several attempts have been made to quantitate cAMP in each cell type throughout development. Tip cells of pseudoplasmodia have been presumed to contain high levels of cAMP because of their ability to induce chemotaxis when placed in fields of amoebae (Rubin 1976). More quantitative studies have shown slightly elevated levels of cAMP in the anterior regions of slugs (Brenner 1977, Pan et al. 1974) while others have shown no detectable differences between front and rear sections (Merkle et al. 1984).

During the culmination stage, however, both the enzymes which regulate cAMP levels as well as cAMP are highly localized. The activity of cyclic AMP phosphodiesterase (PDE), the degradative enzyme, is low in the prespore mass and high within the stalk sheath (Brown and Rutherford 1980). Conversely the cAMP synthetic enzyme adenylate cyclase is high in the prespore mass and low in the prestalk cells (Merkle and Rutherford 1984). As expected from this enzyme distribution, cAMP levels are high in the prespore mass of the culminates and decrease near the basal portion

of the stalk (Merkle et al. 1984). Within the prespore mass cAMP is present in a gradient with higher levels at the base of the mass and lower levels near the tip although the tip itself contains high levels of cAMP (Merkle et al. 1984). In addition, total cAMP levels reach a maximum during the culmination stage (Merkle et al. 1984).

1.3 Cyclic AMP-Dependent protein kinases

The only known mediators of cAMP action in eukaryotic cells are cAMP dependent protein kinase (cAMPdPKs), (reviewed by Krebs and Beavo 1979). These enzymes exist in mammals in the form of a tetramer of two catalytic subunits (C) which possess the phosphotransferase activity and two regulatory subunits (R) which inhibit this activity in the absence but not the presence of cAMP. The enzyme is activated according to the following equation (Beavo et al.1974, Builder et al. 1980a,b):

$$R_2C_2 + 4cAMP \rightleftharpoons R_2 \cdot 4cAMP + 2C$$

The cAMP signal is propagated in the cell by the phosphorylation of the substrates of the catalytic subunit. Most of the known substrates are enzymes whose activities are altered upon phosphorylation (Rubin and Rosen 1975, Lohmann and Walter 1984).

Mammalian cells contain two isoenzyme classes of the cAMPdPK termed type I and type II or PKI and PKII. These classes were initially distinguished by their elution from DEAE resin (Corbin et al. 1975) and have subsequently been shown to have numerous significant differences (Miyamoto et al. 1971, Beavo et al. 1974,1975). The isoenzymes appear to perform different functions in vivo because the relative amounts of the isozymes in mammalian cells vary as a function of tissue type, (Corbin et al. 1975), hormonal stimulation (Lee et al. 1976, Fuller et al. 1978), position in cell cycle (Costa et al. 1976) and degree of differentiation (Schwartz and Rubin 1983) or transformation (Handschin and Eppenberger 1979). A survey of many types of cells under various conditions has led to the recent hypothesis that the type I kinase is predominantly related to cell proliferation and/or the maintenance of the undifferentiated state and the type II kinase is related to cell differentiation (Russell 1978, Handschin and Eppenberger 1979). There are many data that conflict with this hypothesis, however, and the idea is currently the subject of some controversy (Lohmann and Walter 1984).

The existence of a cAMPdPK in Dictyostelium was hypothesized for many years as the importance of cAMP in later development became more and more apparent. An early

report of a cAMPdPK in Dictyostelium was produced by Sampson (1977), but remained unsubstantiated for many years. Several groups of investigators subsequently reported the existence of cAMP binding proteins conjectured to be the regulatory subunit of the kinase (Cooper et al. 1980, deGunzburg and Veron 1981, Leichtling et al. 1981), and finally the holoenzyme form of the cAMPdPK was independently discovered by several groups (Rutherford et al. 1982, deGunzburg and Veron 1982, Cooper et al. 1983, Schoen et al. 1984, Majerfeld et al. 1984).

1.4 Rationale and Objectives

Because cAMP is clearly involved with some aspect of cell differentiation or pattern formation in later stages of Dictyostelium development and because the cAMPdPK is the primary enzyme by which these effects might be mediated, this study was undertaken in an effort to extend our knowledge of this enzyme.

The initial goal of this project was to characterize the enzyme from batch preparations of cells in order to determine its biochemical characteristics, co-factor requirements, optimum conditions for assay, etc., as well as to determine the extent of its similarity to the mammalian isoenzyme forms. Such studies may provide information on possible in vivo regulatory mechanisms which may help

elucidate the function of this enzyme, and comparison with the mammalian isoenzyme forms may provide additional insight as the functions of the mammalian isozymes become increasingly understood.

The second goal of this project was to utilize ultramicromethods to determine the distribution of the cAMPdPK in each cell type at each stage of development. Since cAMP may be directing cells into one or the other pathway of differentiation it was clearly of interest to determine if there is differential distribution of the kinase which may be mediating these effects. Knowledge of the overall levels of the kinase throughout development as well as its cellular distribution may be helpful in elucidating its function and will provide data for comparion with the previously determined distribution of cAMP.

CHAPTER 2

Characterization of a cAMP-dependent protein kinase from Dictyostelium discoideum.

2.1 INTRODUCTION

The cellular slime mold Dictyostelium discoideum is useful as a model system with which to study developmental regulation by adenosine 3'5' monophosphate (cAMP). Upon starvation, Dictyostelium amoebae enter a developmental sequence terminating in the formation of a mature fruiting body consisting of stalk cells supporting a mass of spores (Loomis, 1982). A few hours after depletion of nutrients amoebae begin to aggregate in response to the extracellular chemotactic signal cAMP (Konijn et al., 1968 Bonner et al., 1969). Aside from its role in chemotaxis, cAMP has been described as a regulatory molecule in subsequent stages of development by several investigators. For example, cAMP accumulates during the culmination stage (Brenner, 1978; Pahlic and Rutherford, 1979; Abe and Yanagisawa, 1983) and becomes localized in specific cells of the culminate (Pan et al., 1974; Brenner, 1977; Merkle et al., 1984). Under certain conditions exogenous addition of cAMP causes amoebae to form either stalk cells (Bonner, 1970, Gross et al.,

1976), or spore cells (Town et al. 1976, Kay et al. 1978). During the culmination stage, adenylate cyclase and cAMP phosphodiesterase become localized in prespore and prestalk cells, respectively (Brown and Rutherford, 1980; Merkle and Rutherford, 1984). Prestalk cells from pseudoplasmodia are chemotactically attracted towards cAMP, (Maeda and Maeda, 1974, Sternfeld and David, 1981, Matsukuma and Durston 1979), and adding cAMP exogenously to cells may induce and maintain postaggregation gene expression (Kay, 1979; Landfear et al., 1982).

Clearly, cAMP is involved in processes other than chemotaxis in Dictyostelium. However, little information is available on the mechanism by which the cAMP effects are mediated to cellular metabolism. Although cAMP dependent phosphorylation of Dictyostelium proteins (Lubs-Haukeness and Klein, 1982; Coffman et al., 1982; Frame and Rutherford, 1984), and intracellular soluble cAMP binding proteins (Cooper et al., 1980; De Gunzburg and Veron, 1981; Arents and Van Driel, 1982) have previously been reported, only recently has an early report of an adenosine 3'5' - monophosphate - dependent protein kinase (cAMPdPK) (Sampson, 1977) been confirmed (Rutherford et al., 1982 Leichtling et al., 1982 DeGunzberg and Veron, 1982; Cooper et al., 1983).

All mammalian cAMPdPKs described thus far are composed of catalytic (C) and regulatory (R) subunits. The inactive holoenzyme exists as a tetramer (R_2C_2) and in the presence of cAMP, kinase activity is mediated via the dissociated catalytic subunits. These cAMPdPKs exist in two forms (I and II) which differ in both their physical and kinetic properties (Flockhardt and Corbin, 1982). In comparison to the mammalian cAMPdPKs little is known about the properties of the Dictyostelium enzyme. Moreover, the information which is available comes from studies on relatively crude preparations of the enzyme. In this report we determine some of the characteristics of the enzyme using highly purified preparations of both the holoenzyme and its subunits. Although the regulation of this protein kinase and its role in mediating cAMP dependent phosphorylation during the time course of development is clearly the subject of interest, this question cannot be approached realistically until the optimum assay conditions, co-factor requirements, and behavior of the subunits during chromatography are known.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of cell-free extracts

Growth and differentiation of Dictyostelium discoideum NC4, was carried out as previously described (Rutherford, 1976). At the slug or culmination stage of development the cells were removed from an agar surface with cold distilled water, washed by centrifugation at 1000 x g for 3 minutes, and resuspended in 50 mM tris-HCl buffer (pH 7.5) containing 2 mM mercaptoethanol, 0.02% sodium azide and 2 mM benzamidine (TAMB), (10 ml buffer/gm packed cells). Most of the starting preparations contained 20-30 gm wet weight of cells (approximately 1.5 g protein) in a volume of 200-300 ml. The cells were evenly distributed by two strokes of a Potter-Elvehjem tissue grinder, then were disrupted by three 45 second exposures to a 2 cm probe of a sonic cell disrupter (Model 300, Fisher) at a setting of 45 (125 watts/treatment). The resulting homogenate was centrifuged at 100,000 x g for 60 min and the supernatant removed.

2.2.2 DE 52 chromatography at pH 7.5

Routine preparation of the cAMPdPK was performed by chromatography of extracts on DE 52 cellulose at pH 7.5 and Sephacryl S300 a described in Rutherford et al. (1982, 1984). Briefly, the 100,000 x g supernatant was applied to a

DE-52 cellulose column (1.6 x 13 cm) which had been equilibrated in 50 mM TAMB. The material which did not bind to the resin (flow-through) was allowed to completely elute from the column as determined by the return to the baseline on a column monitor. The active fractions (the flow-through volume) were pooled, precipitated with ammonium sulfate (70% saturation) then dialyzed overnight against 10 mM TAMB.

2.2.3 Sephacryl S-300 column chromatography

The contents of the dialysis bag were then subjected to centrifugation at 9,200 x g for 15 min. The resulting pellet was discarded and the supernantant was applied to a Sephacryl S-300 column (1.6 x 86 cm) which had been equilibrated in 50 mM TAMB. The column was pumped at 30 ml/h with the same buffer and 5 min fractions were collected. Fractions exhibiting cAMP dependent kinase activity were either used directly as a source of enzyme for the characterization studies or were pooled and concentrated by ultrafiltration (PM 10, Amicon). The concentrated S-300 enzyme (3-4 ml) was then subjected to preparative isoelectricfocusing as described below.

2.2.4 Isoelectricfocusing

Further purification of holoenzyme and/or preparation of subunits was performed by isoelectricfocusing (IEF) as

described in Rutherford et al. (1984). IEF was carried out on a horizontal flat bed apparatus in a matrix of granulated gel. The gel matrix consisted of 4% ultrodex (LKB) and 5% Ampholine (either pH 5-8 or a 3:2 mixture of pH 5-8 and pH 7-9 LKB) in a final volume of 100 ml. The slurry was poured onto a glass tray and dried to 65% of its original weight. Focusing was performed for 14-16 h at a constant power of 8W (LKB 2103 Power Supply) with the cathode and anode solutions being 1 M NaOH and 1 M H_3PO_4, respectively. The apparatus (LKB 2117 Multiphor) was cooled at 4° C using a refrigerated circulating water bath. Upon completion of the run, fractions were formed in the gel bed with a metal grid, gel from the fractions was applied to microcolumns and eluted with 3 ml of TAMB. The eluted fractions were then assayed for cAMPdPK and cAMP binding activity as described below.

2.2.5 Gel filtration chromatography

The molecular weight of the holoenzyme was determined by applying a 1.0 ml sample of either an S-300 or an IEF peak cAMP dependent fraction to a Sephadex G-200-40 (superfine) column (1.6 x 94 cm) which had been equilibrated in 50 mM TAMB plus 100 mM KCl. The column was pumped at 3.5 ml/h with the same buffer and 16 min fractions (approximately 1 ml each) were collected. The regulatory and catalytic subunit molecular weights were determined by

chromatography on a Sephadex G-100 column (1.6 x 92 cm) which had been equilibrated in 50 mM TAMB. The column was pumped at 12 ml/h and 15 min fractions (3 ml each) were collected. The columns were calibrated with the molecular weight markers indicated in the figure legend. Kemptide kinase and cAMP binding activities of each fraction were determined as described below.

2.2.6 Cyclic AMP affinity chromatography

Further purification of the regulatory subunit was performed by chromatography on cAMP affinity resin. N6-cAMP-ethane-agarose (P.L. Biochemicals) was equilibrated in TAMB containing 5 mM 5'AMP and one ml of resin was placed in a 1.0 x 10.0 cm column. Samples of holoenzyme (S300 or IEF) or regulatory subunit preprations of up to 100 ml could be applied to this resin with complete retention of cAMP binding activity. The non-specific, high molecular weight cAMP binding protein (see discussion) was prevented from binding to the resin by the inclusion of 5'AMP in the column buffer. After application of the sample the column was washed with several column volumes of TAMB, then washed with TAMB containing 2 M NaCl to remove non-specifically bound proteins. Washing was continued until the absorbance reading on the column monitor returned to baseline, then the column was re-washed with the original column buffer to

remove the NaCl. To elute the regulatory subunit the resin was incubated at 4°C for 30 minutes with 5 ml TAMB containing 1 mM cAMP. Treatment of the resin with additional elution buffer did not release further activity, nor did performing the elution at room temperature increase the recovery. To remove the cAMP from the regulatory subunit, the eluted sample was chromatographed on DE 52 resin pH 7.5. At this pH the regulatory subunit bound to the resin while the cAMP did not bind (determined by monitoring absorbance). The DE 52 column was washed overnight with TAMB to allow time for the bound cAMP to be released from the regulatory subunits. The regulatory subunit activity could then be eluted, free from cAMP, with a linear gradient of 0-0.3 M KCl.

2.2.7 Reconstitution of Holoenzyme

Samples of catalytic and regulatory subunits were mixed together at various relative concentrations in a final volume of 25 µl, preincubated for 15 minutes and then assayed for Kemptide kinase activity in the presence and absence of cAMP. Control samples of catalytic subunit were diluted with equal volumes of TAMB prior to assay. The regulatory subunit preparations used were also assayed for kinase activity and when any was present, this value was subtracted from the activity of the combined samples.

2.2.8 Protein kinase and cAMP binding assays

Protein kinase activity was assayed in a total volume of 50 µl with 25 µl of the enzyme sample and 25 µl of a reaction mixture which contained 50 mM potassium phosphate buffer (pH 6.5), 3 mM dithiothreitol, 10 mM $MgCl_2$, 35 µM Kemptide, and 25 µM [γ-^{32}P]ATP (0.4 Ci/mmol) either with or without 20 µM cAMP. After a 15 minute incubation at 25°C the entire reaction volume was removed to a 1 cm square piece of Whatman P81 filter paper. Papers were immediately placed in ice-cold 30% acetic acid for 5 min, and were transferred through additional 5 min washes of ice-cold 30% and 15% acetic acid, and room temperature 15% acetic acid acid to stop the reaction and to remove the unbound ATP from the filter papers. The filters were dehydrated in acetone, then dried for determination of radioactivity. Kemptide was used as substrate unless otherwise indicated. The amino acid sequence of Kemptide is leu-arg-arg-ala-ser-leu-gly.

Cyclic AMP binding activity was measured in a total volume of 125 µl containing 100 µl of the protein sample and 25 µl of a reaction mixture containing 25 mM dithiothreitol, 150 mM [2,8-^{3}H]cAMP (130 Ci/mmole) in 50 mM tris-HCl buffer (pH 7.5). After incubation for 5 min the entire reaction mixture was removed to a Hoefer filter reservoir containing 5 ml of ice cold 50 mM tris-HCl (pH

7.5). The solution was immediately filtered through a Gelman GN-6 filter (0.45 µm) by vacuum filtration. The filter was washed twice in the same buffer then removed and dried for determination of radioactivity.

2.3 RESULTS

2.3.1 Molecular weight

The cAMPdPK holoenzyme from Dictyostelium has been reported to elute from gel filtration columns at a position corresponding to molecular weights greater than 500,000 (Rutherford et al., 1982), 270,000 (Schoen et al., 1984), 230,000 (Rutherford et al., 1984) 160,000-180,000 (Majerfeld et al., 1984) and 82,000-88,000 (De Gunzburg et al., 1984). A possible source of the variability in the reported molecular weights of the holoenzyme could be the use of relatively crude preparations of the enzyme. In such preparations the enzyme may form aggregates or complex with its protein substrates. Such substrates are known to co-elute from gel filtration columns along with the holoenzyme (Frame and Rutherford, 1984). In addition, it has recently been found that a Kemptide phosphatase is present in crude preparations of the kinase and elutes from gel filtration columns at a position where it overlaps that of the cAMPdPK.

(Ferris et al. manuscript in preparation). Thus, the activity of the kinase is masked in fractions which contain the phosphatase due to dephosphorylation of the product of the kinase reaction. Because the kinase activity could only be detected in fractions that did not contain the phosphatase, the molecular weight of the holoenzyme was initially overestimated (Rutherford et al. 1982). Figure 2A shows that when the holoenzyme was partially purified through the steps of DE 52, S300, and IEF before chromatography on Sephadex G-200-40, cAMP dependent kinase activity was found in fractions corresponding to a molecular weight of 190,000.

The molecular weights of the catalytic and regulatory subunits were determined by Sephadex G-100 column chromatography. The catalytic subunit was prepared by passing the holoenzyme through DE-52 cellulose and Sephacryl S-300 chromatography, then dissociating the subunits during preparative isoelectric focusing. The regulatory subunit was prepared by chromatography of the holoenzyme on DE-52 cellulose and Sephacryl S-300, followed by dissociation of the subunits on cAMP-affinity chromatography. Free regulatory subunit could also be recovered from the KCl elution of the DE-52 cellulose step. To ensure that the preparations contained the actual catalytic and regulatory

subunits the samples were mixed and then tested for reconstitution of the holoenzyme (Table 1). Regardless of the method employed in preparation of the subunits, the regulatory subunit consistently eluted in fractions corresponding to a Mr of 49,000 and the catalytic subunit Mr of 40,000 (Fig. 2B). These molecular weight determinations are consistent with an R_2C_2 subunit arrangement, similar to the mammalian enzymes, as has been previously proposed for Dictyostelium (Majerfeld et al., 1984).

2.3.2 Cyclic AMP affinity chromatography

The subunit nature of the holoenzyme could be demonstrated by chromatography of the S300 or IEF kinase samples on cAMP agarose. When cAMP-dependent kinase activity was applied to the resin, non-cAMP dependent kinase activity (the catalytic subunit) eluted in the flow through volume while the cAMP binding activity (the regulatory subunit) was retained (data not shown). After elution of the regulatory subunit from the resin and removal of the cAMP, the two subunit activities could be reconstituted into holoenzyme activity.

Although chromatography of the regulatory subunit on the cAMP agarose resulted in substantial purification (approximately 4,000 fold), the protein was not homogenous as judged by polyacrylamide gel electrophoresis (silver

stain). In addition, yields from cAMP chromatography were low (1-10%), and the activity of the protein was unstable after this treatment. Therefore, for most experiments other procedures were used to prepare the regulatory subunit (Rutherford et al. 1984). However, this was an easy and rapid method for obtaining free catalytic subunit from holoenzyme samples.

2.3.3 Reconstitution of holoenzyme

Table 1 shows the results of three typical reconstitution experiments. The catalytic subunit alone exhibited the same activity in the presence and absence of cAMP. When the regulatory subunit was added the kinase activity was inhibited in the absence of cAMP, but addition of cAMP to the mixture relieved this inhibition. It was generally necessary to concentrate the regulatory subunit several fold in order to obtain such results. The experiments shown were performed using Kemptide as the substrate. Very little to no subunit reassociation could be produced if histone rather than Kemptide was used as the substrate.

2.3.4 Substrate specificity

The substrate specificity and cAMP dependence of IEF purified holoenzyme is illustrated in Table 2. Kemptide was

the best substrate for cAMPdPK and was phosphorylated with the most cAMP dependence. Histones VII-S and VI-S were less efficient substrates and displayed a lower degree of cAMP dependence, while essentially no phosphorylation occurred with histone II-S, casein or protamine sulfate.

2.3.5 Effect of pH on the activity of cAMPdPK and its subunits

To determine the pH optimum for the kinase and the cAMP binding activities, samples were purified through IEF and extensively dialyzed in buffers at various pHs. Activity was then assayed with a reaction mixture made with buffers of the appropriate pH (for details see legend Fig. 2). The cAMPdPK showed maximum activity and cAMP dependency at pH 7.5. Cyclic AMP dependent activity decreased as pH varied from 7.5 and was totally lost at pH 5 and 10 (Fig. 3A). The isolated catalytic subunit had the same pH activity profile as the holoenzyme (Fig. 3B). The cAMP binding activity of the regulatory subunit was essentially constant over the pH range of 5-10 (Fig. 3C).

2.3.6 Ion requirements of cAMP dependent kinase and cAMP binding activities

Analyses of ion requirements and kinetic properties of the cAMPdPK were performed in collaboration with Dr. Michel

Cloutier. It was found that the activity of the holoenzyme was negligible in the absence of divalent cations and was maximum with Mg^{2+} (Fig. 4). Among the other ions examined, only Mn^{2+} supported significant kinase activity but was four fold less effective than Mg^{2+}. However, the cAMP binding of the regulatory subunit did not require divalent cations, nor was there any appreciable difference in this activity in the presence of any of the ions tested.

2.3.7 Kinetic properties of the cAMPdPK and its subunits

Figure 5 illustrates the time course of the kinase activity of the holoenzyme and catalytic subunit and the cAMP binding activity of the regulatory subunit. The kinase activity of the holoenzyme was linear for 30 minutes while the catalytic subunit activity was linear for 60 minutes. The cAMP binding activity of the regulatory subunit was virtually instantaneous, with maximum binding occurring within 30 seconds and remaining constant through 60 minutes. In one experiment, incubation was maintained for 3 h with no change in the cAMP binding.

Figure 6 illustrates the Scatchard analysis (Scatchard, 1949) for cAMP binding of both the holoenzyme and the isolated regulatory subunit. Regression analysis of the data points produced identical cAMP dissociation constants (Kds) of 28 nM for both preparations. The Michaelis

constants for Kemptide and ATP for the holoenzyme were determined to be 15 μM for ATP and 75 μM for Kemptide (data not shown). Identical values were obtained for the isolated catalytic subunit.

2.4 DISCUSSION

In this report we extend our earlier descriptions of a cAMPdPK from D. discoideum (Rutherford et al. 1982, 1984) and describe some physical and kinetic properties of the holoenzyme and its isolated regulatory and catalytic subunits. Proof that the kinase and cAMP binding activities prepared and utilized for this study were the catalytic and regulatory subunits of the holoenzyme was provided by reconstitution experiments published elsewhere (Rutherford et al. 1984) and in Table 1.

In addition to the regulatory subunit, a second cAMP binding protein was detected (not shown). This activity eluted from the DE-52 column in the salt gradient and when applied to the G-100 column eluted in the void volume, well separated from the regulatory subunit. This high molecular weight preparation displayed a slow time course of cAMP binding and was also capable of binding 5'AMP. These properties are similar to those of a previously described cAMP binding protein (Veron and Patte, 1978; Leichtling et

al., 1981; Arents and Van Driel, 1982) which has since been shown to carry S-adenosyl-L-homocysteine hydrolase activity (DeGunzburg et al. 1983). These activities were not found in association with the holoenzyme preparation and were not pursued further.

Holoenzyme purified through isoelectricfocusing used in this and in a previous report (Rutherford et al., 1984) is the most highly purified cAMPdPK preparation from D. discoideum reported to date. Holoenzyme purified through isoelectricfocusing is not homogeneous as determined by SDS polyacrylamide gels but has been purified approximately 600 fold. Attempts to further purify the holoenzyme by conventional methods developed for the purification of cAMPdPK from rabbit muscle and other sources were unsuccessful (Rutherford et al., 1984).

The subunit nature of the holoenzyme was easily demonstrated by a variety of methods, including cAMP affinity chromatography, IEF, chromatofocusing, histone affinity, etc. (Rutherford et al., 1984). These purified subunits were free of endogenous substrates and gave only a few bands on silver stained SDS polyacrylamide gels (data not shown). The physical and kinetic data for the catalytic and regulatory subunits reported here were identical regardless of the method of preparation of the subunits.

Determination of the molecular weights of the regulatory and catalytic subunits, 49,000 and 40,000 respectively (Fig. 2B), are highly reproducible regardless of the method of preparation of the subunits. Because of the nature of gel filtration chromatography these values are probably overestimations of the molecular weights. A regulatory subunit of 41,000 Mr has been reported based on photoaffinity labelling followed by SDS polyacrylamide gel electrophoresis (Majerfeld et al 1984).

An accurate molecular weight for the holoenzyme has been more difficult to obtain. For example, if the flow through portion of the DE-52 cellulose column was concentrated by ultrafiltration and applied to a Sephacryl S-300 column, cAMP dependent kinase activity eluted at a volume corresponding to a molecular weight of at least 500,000 (Rutherford et al., 1982). However, if the DE-52 activity was precipitated with ammonium sulfate prior to S-300 column chromatography, the activity consistently eluted with the catalase marker (Mr 232,000) (Rutherford et al., 1984). These values are significantly greater than those of the corresponding mammalian enzymes (Flockhardt and Corbin, 1982). However, a number of endogenous substrates co-elute from the S-300 column with cAMPdPK (Frame and Rutherford 1984), and it is possible that an enzyme-

substrate and/or self-aggregation event occurred in these crude preparations. In addition the presence of the Kemptide phosphatase may have obscured the true size of the holoenzyme.

In any event, further purification of the Dictyostelium enzyme yielded a preparation which more closely resembled the mammalian enzyme with respect to the molecular weight. When the activity which eluted from the S-300 column was first subjected to IEF, then applied to a Sephadex G-200-40 gel filtration column, cAMP dependent kinase activity eluted in fractions corresponding to a molecular weight of 170,000 to 190,000 (Fig. 2A). These values are more similar to the mammalian Mr's and are consistent with the proposed R_2C_2 subunit structure for the D. discoideum holoenzyme (Majerfeld et al., 1984)

Reconstitution of holoenzyme was performed by titrating the catalytic subunit with increasing concentrations (constant volumes) of regulatory subunit. Since we were unable to purify either subunit to homogeneity it was not possible to calculate molar ratios of the subunits during reconstitution or to determine the stoichiometry of the activation reaction. Because the regulatory subunit tended to be more labile during purification it was generally necessary to perform considerable concentration of the

sample to obtain adequate activity to regulate the catalytic subunit.

Reconstitution of holoenzyme was obtained with subunit activities from every purification procedure that we utilized, thus ensuring that we were not characterizing a different kinase or cAMP binding activity. The ability to obtain subunit reassociation was related to the substrate used. Very little to no reconstitution was obtained when histone VII-S was used as the substrate whereas it was relatively easy to produce reconstitution in the presence of Kemptide. As shown in Table 2 when Kemptide was the substrate for native enzyme there was more cAMP-dependency (lower activity ratio) than when histone VII-S was the substrate. This effect might be produced if histone was inducing the subunits to dissociate as it does to the mammalian type I enzyme (Beavo et al. 1975). Although further work would be necessary to establish this as a mechanism with the Dictyostelium enzyme, it would explain the difficulty in producing subunit reassociation in the presence of histone.

Characterization of the holoenzyme shows that the activity has a pH optimum of 7.5, is highly dependent on Mg^{2+}, and displays linear kinetics. Since the cAMP binding activity of the isolated regulatory subunit is not dependent

on pH, divalent metal ions, or incubation time, these alterations of the holoenzyme kinase activity are specific characteristics of the catalytic subunit.

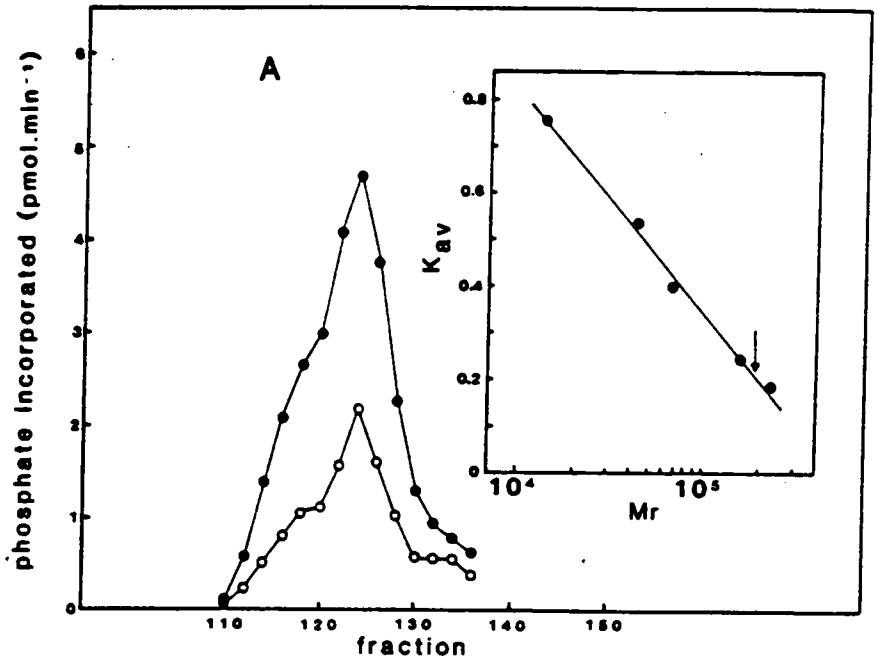
The holoenzyme and the isolated catalytic subunit displayed identical ATP and Kemptide Km's while the cAMP Kd was the same for the holoenzyme and the isolated regulatory subunit. These results suggest that the mechanism of action of cAMP with respect to activation of the kinase is at the level of maximum velocity, rather than by a change in the affinity for its substrates.

The cAMPdPK from D. discoideum has been partially purified and its chromatographic, (Rutherford et al., 1984) physical and kinetic properties described. The enzyme is remarkably similar to cAMPdPKs of mammalian origin in many of the properties reported here and in Rutherford et al. (1984), such as subunit composition, Kms, preference for basic substrates, affinity for and activation by cAMP, etc. However the fact that the Dictyostelium enzyme behaves considerably differently from the mammalian enzyme during chromatographic procedures indicates a certain amount of dissimilarity. Determining whether or not this enzyme performs the same metabolic functions as the mammalian enzyme will require further investigation.

Figure 2. Molecular weight determination of the cAMPdPK and its subunits.

(A) Gel filtration chromatography of the cAMPdPK on Sephadex G-200-40. Kemptide kinase activity with (—●—) and without (—O—) 20 μM cAMP. Insert shows a molecular weight calibration using ribonuclease A (13,700), ovalbumin (43,000), bovine serum albumin (67,000), aldolase (158,000), and catalase (232,000). The arrow indicates the position of the peak cAMP dependent Kemptide kinase activity (190,000). The sample that was applied to this column had been purified through S 300.

(B) Gel filtration chromatography of the catalytic and regulatory subunits on Sephadex G-100. cAMP bound by the regulatory subunit (—O—), Kemptide kinase activity by the catalytic subunit (—●—). Insert shows a molecular weight calibration using ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000). Arrow a indicates the position of the peak cAMP binding activity (49,000). Arrow b indicates the position of the peak Kemptide kinase activity (40,000).


A
phosphate incorporated (pmol.min⁻¹)
fraction
Kav
Mr
10⁴
10⁵

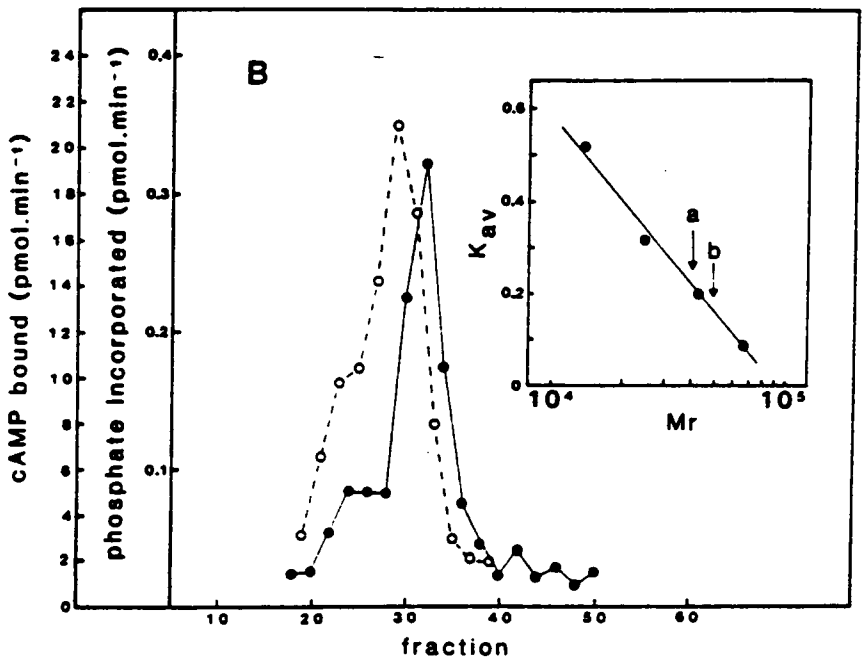

B
cAMP bound (pmol.min⁻¹)
phosphate incorporated (pmol.min⁻¹)
fraction
Kav
Mr
a
b
10⁴
10⁵

Table 1 Reconstitution of holoenzyme activity from regulatory and catalytic subunits.

CONTENTS[1]	cAMP	enzyme activity[2]	%control
catalytic subunit	+/-	9835	100
cat + reg	-	1404	14
cat + reg	+	9492	97
catalytic subunit	+/-	11532	100
cat + reg	-	2568	22
cat + reg	+	12532	110
catalytic subunit	+/-	9828	100
cat + reg	-	2829	29
cat + reg	+	9707	99

[1] Catalytic and regulatory subunit samples were prepared from IEF or DE 52 pH 8.5. The protocol for the experiment is described in the text (section 2.2.7.).

[2] Activity is expressed as cpm ^{32}P incorporated into Kemptide.

Table 2. Substrate specificity of cAMP-dependent holoenzyme.

Substrate[1]	cAMP	enzyme[2] activity	cAMP[3] dependency	activity[4] ratio
Kemptide	+	1.64		
	-	.27	6.1	.16
Histone VII-S	+	.29		
	-	.17	1.7	.59
Histone VI-S	+	.10		
	-	.05	2.0	.50
Histone II-S	+	N.D.[5]		
	-	N.D.	-	-
Casein	+	N.D.		
	-	N.D.	-	-
Protamine sulfate	+	N.D.		
	-	N.D.	-	-

[1] The substrate concentrations used were; Kemptide 58 ug/ml, histones 1 mg/ml, casein 2.5 mg/ml, protamine sulfate 1 mg/ml.

[2] Enzyme activity is expressed as phosphate incorporated into substrate (pmole/min).

[3] cAMP dependency is defined as the enzyme activity in the presence of cAMP divided by the activity in the absence of cAMP.

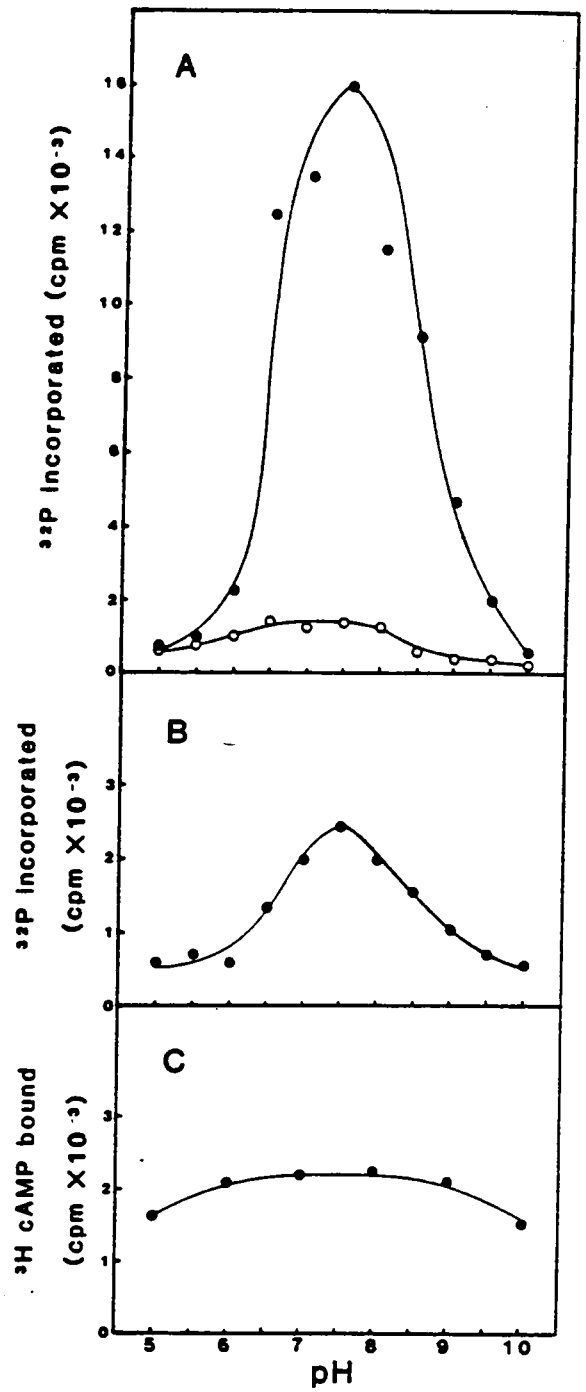
[4] Activity ratio is defined as the activity in the absence of cAMP divided by the activity in the presence of cAMP.

[5] N.D. = not detectable (< 0.05 pmole phosphate incorporated/min).

Figure 3. Effect of pH on the activity of cAMPdPK and isolated catalytic and regulatory subunits.

(A) Protein kinase activity of cAMPdPK with (—●—) and without (—○—) 20 μM cAMP. (B) Protein kinase activity of the isolated catalytic subunit. (C) Cyclic AMP binding activity of the isolated regulatory subunit.

All preparations were purified through IEF. Three buffer systems were used to cover the indicated pH range; potassium citrate pH 5.0-6.0, potassium phosphate pH 6.0-8.0, and potassium borate pH 8.0-10.0. Enzyme samples were dialyzed overnight vs 5 mM buffer at pH 5.5, 7.0 and 9.0 in citrate, phosphate and borate buffers, respectively. Kinase reaction mixtures were prepared with 50 mM buffer at each of the indicated pH values so that the final pH in the assay tube was essentially equivalent to that of the reaction mixture. For the binding assay, reaction mixture was prepared with 250 mM buffer. Both holoenzyme and catalytic subunit demonstrated a kinase activity pH optimum of 7.5, cAMP binding activity was not significantly affected by pH over the range tested.


A
B
C
^{32}P incorporated (cpm X10^{-3})
^{32}P incorporated (cpm X10^{-3})
^{3}H cAMP bound (cpm X10^{-3})
pH
5
6
7
8
9
10

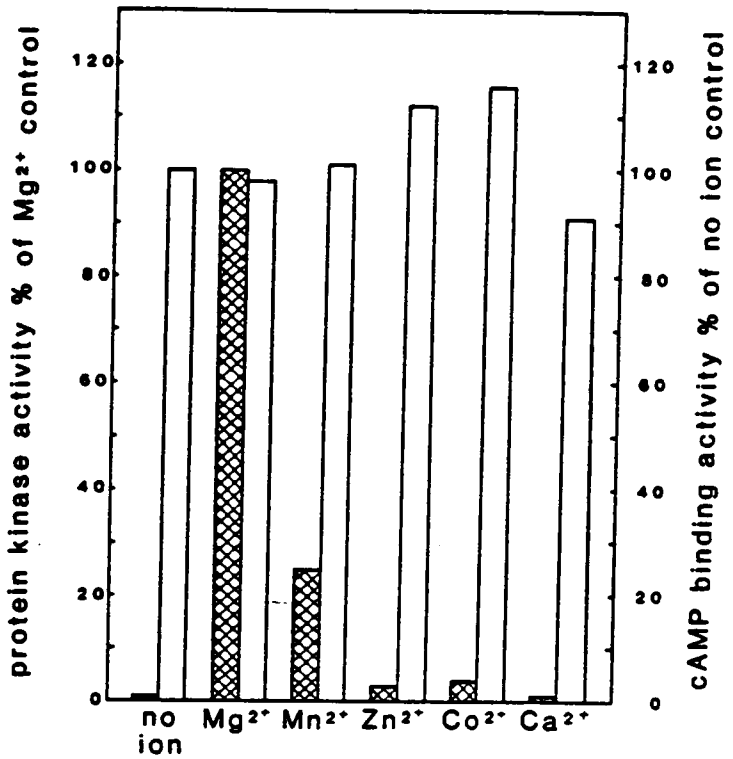


Figure 4. Effect of divalent cations on cAMPdPK activity and cAMP binding activity of the regulatory subunit. Protein kinase activity is expressed as the percent of activity in the presence of Mg^{2+} (cross hatched bars). Cyclic AMP binding activity is expressed as the percent of activity without added metal ions (open bars). Divalent cations were added as chloride salts at a final concentration of 5 mM for the kinase assay and 10 mM for the binding assay. The kinase reaction mixture contained 0.8 mg/ml Histone VII-S as substrate and 20 µM cAMP.

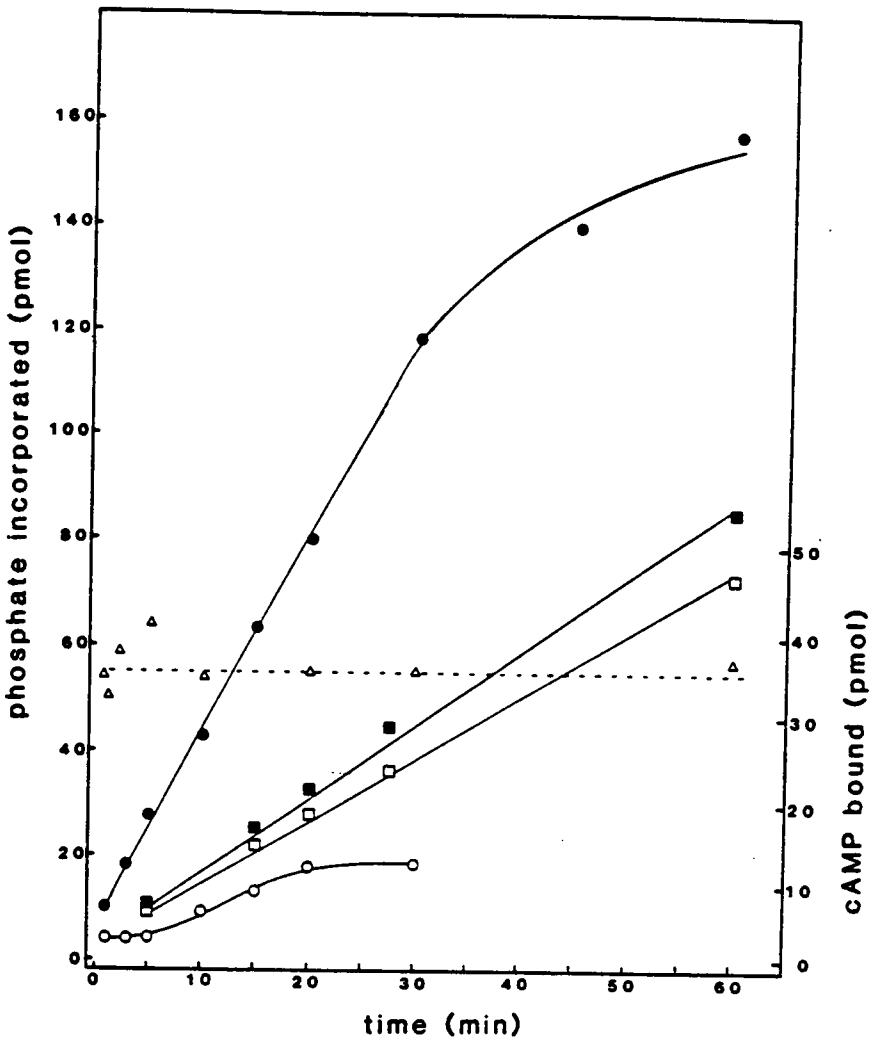


Figure 5. Time course of the cAMP dependent holoenzyme and catalytic subunit kinase activities and the regulatory subunit cAMP binding activity. Kinase activity of the holoenzyme (● ○) and the catalytic subunit (■ □) with (● ■) and without (○ □) 20 µM cAMP. Cyclic AMP binding activity of the regulatory subunit (△). For the kinase assay the substrate was 35 µM Kemptide for IEF purified holoenzyme and 0.8 mg/ml Histone VII-S for catalytic subunit purified through chromatofocusing and histone affinity column chromatography (Rutherford et al., 1984).

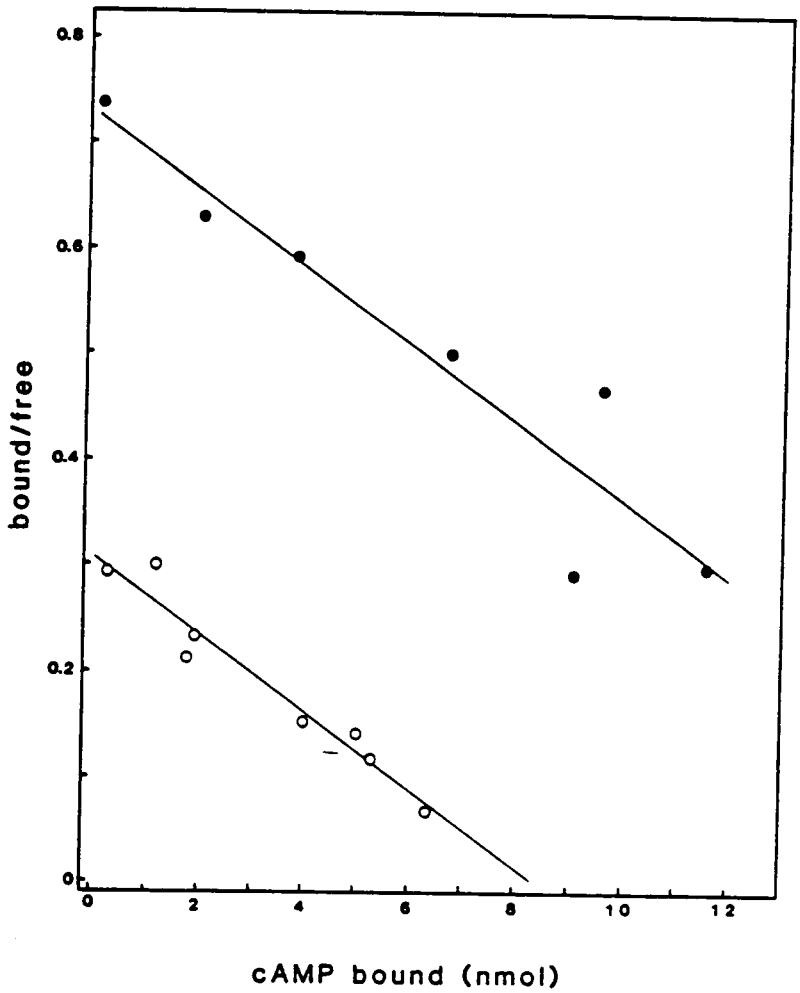


Figure 6. Scatchard analysis of cAMP binding. Reaction mixtures were prepared with concentrations of [^{3}H]cAMP ranging from 0.1 to 100 nM. Samples were incubated on ice for 60 minutes. Each data point represents the average of triplicate determinations which varied by less than 10%. Dissociation constants were determined by regression analysis of the data points. A cAMP Kd of 28 nM was determined for both holoenzyme (—O—) and isolated regulatory subunit (—●—). Data generated according to the method of Scatchard (1949).

CHAPTER 3

Association and dissociation properties of a cAMP dependent protein kinase from Dictyostelium discoideum.

3.1 INTRODUCTION

The cellular slime mold Dictyostelium discoideum offers an excellent model system for the study of development. When deprived of nutrients the unicellular amoebae enter a sequence of events resulting in mass of spores supported by a moribund stalk (Loomis 1982). A variety of techniques has implicated adenosine 3'5' cyclic monophosphate (cAMP) as a regulatory molecule in this developmental process. Exogenously applied cAMP causes both wild type and mutant cells to differentiate into either stalk or spore cells depending on the conditions (Bonner 1970, Town et al. 1976, Gross et al. 1976, Kay et al. 1978). After separation of the cell types on density gradients, the prestalk cells are more responsive than prespore cells to added cAMP (Maeda and Maeda 1974). In cell clumps embedded in agar the the prestalk cells move towards a source of cAMP (Sternfeld and David 1981), and in mixtures of vitally stained cells, the prestalk cells stream toward a source of cAMP (Matsukuma and Durston 1979). During the culmination stage, adenylate cyclase and cAMP phosphodiesterase become localized in

prespore and prestalk cells, respectively, (Brown and Rutherford 1980, Merkle and Rutherford 1984). In addition, cAMP accumulates during this stage (Brenner, 1978, Pahlic and Rutherford, 1979, Abe and Yanagisawa 1983) and becomes localized in one cell type (Pan et al. 1974, Brenner 1977, Merkle et al. 1984). Adding cAMP exogenously to cells may induce and maintain post aggregation gene expression (Kay 1979, Landfear and Lodish 1982).

The only known mediators of cAMP action in eukaryotic cells are cAMP dependent protein kinases. This laboratory (Rutherford et al. 1982,1984) and others (deGunzburg and Veron 1981,1982, Majerfeld et al. 1982,1984, Cooper et al. 1983, Schoen et al. 1984) have demonstrated existence of a cAMPdPK in Dictyostelium and reported many of its biochemical properties. In this study we extend our characterization of the Dictyostelium cAMPdPK with respect to two major areas; association and dissociation properties of the subunits, and comparison to the mammalian prototype isoenzyme forms.

In mammalian cells cAMPdPKs exist in two forms, types I and II. Both types are tetramers of two catalytic and two regulatory subunits (R_2C_2) and are activated via dissociation of the subunits by cAMP according to the following equation (Beavo et al. 1974, Builder et al. 1980a,b)

$$R_2C_2 + 4cAMP \rightleftharpoons R_2 \cdot 4cAMP + 2C$$

The catalytic subunits of both types are structurally and functionally similar (Betchel et al. 1977). However, the isoenzymes exhibit significant differences in their chromatographic behavior on DEAE cellulose (Corbin et al. 1975), molecular weights of the regulatory subunits (Hofmann et al. 1975), subunit association and dissociation properties (Haddox et al. 1972, Tao 1972, Beavo et al 1975), and effects of MgATP (Beavo et al. 1974, 1975). Because the levels of the isozymes fluctuate with respect to physiological parameters such as position in cell cycle (Costa et al. 1983), state of differentiation (Schwartz and Rubin 1983), and state of transformation (Handschin and Eppenberger 1979), it is believed that the isozymes have different and specific roles relating to the biochemistry of these processes. Although the nature of these roles has not yet been determined, it has been postulated (Russell 1978, Handschin and Eppenberger 1979) that the type I enzyme is involved with cell proliferation and the type II enzyme is involved with differentiation.

In view of the facts that in Dictyostelium there is no evidence of multiple forms of this enzyme, the major phase of proliferation is temporally distinct from differentiation

(Raper 1941, Bonner 1947) and cAMP appears to play an important role in differentiation, it was of interest to characterize the Dictyostelium cAMPdPK with respect to the parameters that distinguish between the mammalian isozyme forms.

In addition we investigated the conditions under which the enzyme will dissociate into its subunit components and reassociate into the holoenzyme form. Because the overall state of activity of the enzyme depends upon the equilibrium position of the above reaction, knowledge of the properties which promote or inhibit dissociation or reassociation of the subunits will be useful in attempting to understand the physiological effects of this enzyme in Dictyostelium.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of cell-free extracts

Growth and differentiation of Dictyostelium discoideum NC4, was carried out as previously described (Rutherford 1976).

3.2.2 Preparation of holoenzyme

Preparation of cAMP-dependent protein kinase activity was performed by chromatography of cellular extracts on DE 52 pH 7.5 and Sephacryl S300 as described in Rutherford et

al. (1982, 1984). In some cases this was followed by further purification by isoelectric focusing as in Rutherford et al. (1984).

3.2.3 Preparation of Subunits

Catalytic and regulatory subunit preparations for reconstitution experiments and density gradient centrifugation were prepared either by chromatography on DE 52 pH 8.5 or by isoelectricfocusing as described in Rutherford et al. (1984). Before the experiments samples were dialyzed in TAMB to adjust the pH and remove either KCl or ampholytes.

3.2.4 Density Gradient Centrifugation

Sedimentation studies were performed in linear gradients of 5-17% glycerol made in 50 mM TAMB (12.8ml). Protein samples of 0.5 ml were layered onto the gradients which were centrifuged for 24 h at 100,000 x g in a Beckman SW 41 Ti rotor. After centrifugation the gradients were immediately fractionated with an ISCO model 640 density gradient fractionator. Six drop fractions were collected from the top of the gradients, and were then assayed for cAMPdPK and/or cAMP binding activity as described below. Protein standards were included in every experiment and their sedimentation was determined by monitoring the

ultraviolet absorption of the gradient fractions with an ISCO Model UA 50 absorption monitor.

3.2.5 Protein kinase and nucleotide binding assays

Protein kinase activity and cAMP binding were assayed as described (Rutherford et al. 1984). ATP binding was assayed by the same procedure used for cAMP binding with the substitution of 150 nM [2,8-^{3}H]ATP (30 Ci/mmole) for the cAMP and the inclusion of 2 mM $MgCl_2$ in the reaction mixture.

3.3 RESULTS

3.3.1 DISSOCIATION PROPERTIES

Previous attempts to demonstrate dissociation of the Dictyostelium holoenzyme by cAMP using chromatographic techniques or isoelectric focusing were unsuccessful (Rutherford et al. 1984). However, as shown in Figure 7, it was possible to demonstrate cAMP-induced dissociation by utilizing density gradient centrifugation. In the control experiments holoenzyme and free catalytic and regulatory subunit activities were separately sedimented through density gradients (Fig. 7A,B). In seven separate experiments with gradient tubes run in duplicate, holoenzyme activity was recovered near the BSA marker with an average

sedimentation coefficient (S20,w) of 4.22 (Fig. 7A). Cyclic AMP binding activity in these experiments exactly paralleled the protein kinase activity. These results were obtained whether the holoenzyme was obtained from S300 or IEF. Centrifuging for longer periods of time (up to 40 h) or through shallower gradients (5-13%) caused the molecular weight standards to spread out, but did not result in the holoenzyme activity sedimenting any further. Neither were any differences from these results observed when samples were concentrated by ultrafiltration (Amicon PM 10) up to 4-5 fold before application to the gradient or when EDTA, DTT, or MgATP were included in the gradients. However, centrifugation at higher speeds (41,000 rpm) tended to result in loss of cAMP dependency and recovery of the kinase activity in the same position as free catalytic subunit. As shown in Figure 7B, free catalytic and regulatory subunit activities sedimented in fractions corresponding to S20,w values of 2.7 and 2.3, respectively. Yields from these experiments ranged from 20-100%. In contrast to the gel filtration molecular weight determinations, the results from these experiments are consistent with a dimeric holoenzyme structure.

Demonstration of dissociation by cAMP was accomplished by sedimenting a holoenzyme sample through a gradient

containing either 125 nM ^{3}HcAMP or 20 μM cAMP. Under either of these conditions protein kinase activity sedimented to the same position as catalytic subunit and when ^{3}HcAMP was used, cAMP binding activity was also recovered at the lower sedimentation value (Fig. 7C). These results clearly demonstrate dissociation of the subunits by cAMP. Although the shift from holoenzyme to subunits was small in terms of number of fractions it was reproducible over the course of the fourteen experiments. Inclusion of NaCl was not required to produce dissociation nor did it affect the sedimentation of the holoenzyme or the subunits (not shown).

Histone was also tested for its ability to produce dissociation of the Dictyostelium enzyme. In a preliminary experiment we analyzed the effects of histone VII-S and Kemptide on the activity ratio of the Dictyostelium holoenzyme (Fig. 8). When the enzyme was assayed with each substrate separately the activity ratio was much greater with histone than with Kemptide. When the enzyme was assayed in the presence of both substrates the resultant activity in the presence of cAMP was additive. However, the activity in the absence of cAMP was was greatly increased compared to that value obtained with Kemptide alone or the additive value of both substrates. The net result therefore was an activity ratio equivalent to that observed with

histone alone. An obvious explanation for this result is that histone is promoting dissociation of the regulatory from the catalytic subunits.

Included in this experiment was a time course of the preincubation with histone because the mammalian type I isozyme requires a preincubation period for the expression of histone effects. With the Dictyostelium enzyme there was no difference in activity ratios based solely upon preincubation intervals, and the activation effect produced by histone occurred instantaneously, (i.e., without preincubation). This instantaneous activation occurred even in the presence of MgATP, which inhibits histone-induced activation of PKI (Beavo et al. 1975). Preincubation with Kemptide alone for 30 minutes had no effect on the activity ratio.

Because these results could be explained by assuming that histone is inducing the subunits to separate, we continued the investigation of histone effects by performing centrifugation of the Dictyostelium holoenzyme through histone-containing density gradients. Surprisingly, although the kinase activity recovered was not cAMP-dependent it nevertheless sedimented to the same position as cAMP-dependent holoenzyme (Fig. 7D). Cyclic AMP binding activity, albeit at low levels, paralleled the peak of

peak of protein kinase activity in these gradients, so that there was no evidence of a shift in sedimentation of either of the subunits. These results were obtained a total of four times. The mechanism by which histone can activate the holoenzyme without causing dissociation is unknown and will require further experimentation to determine.

3.3.2 Time course of reconstitution

The reassociation of the mammalian subunits can be affected by a variety of factors such as MgATP, ionic strength, and pH. The effect of MgATP is to facilitate the reassociation of the type I subunits after the removal of cAMP. In the absence of MgATP the type I subunits reassociate slowly or not at all, while the type II subunits reassociate instantly under either condition. (Haddox et al. 1972, Beavo et al. 1974,1975). To study the time course of reconstitution as well as the effects of MgATP on this process with the Dictyostelium enzyme, two approaches were used. In the first, holoenzyme was incubated with 0.1 M NaCl and 20 µM cAMP to dissociate the subunits (Rutherford et al. 1984), then the sample was passed though a 1.5 x 5.5 cm column of G25 resin (PD 10, Pharmacia) to remove the cAMP. The NaCl was included to prevent interactions of the subunits with the resin. Approximately 2 min elapsed between loading and elution of the sample. Aliquots of

enzyme were assayed for cAMPdPK activity at various intervals after elution from the column. To minimize possible effects of the MgATP in the reaction mixture reaction times were kept as short as possible (2 minutes). As shown in Figure 9, the activity ratios before the treatment and immediately after elution from the column were equal, indicating that the catalytic and regulatory subunits had reassociated to their fullest extent by 2 minutes after removal of cAMP.

In order to verify that the results of this experiment were not affected by interactions of the enzyme with the resin, (see discussion) this experiment was also performed another way. This second method also allowed a more precise determination of the time required for reassociation since there was no lag time while the enzyme passed through the column. In this approach the holoenzyme was mechanically dissociated by chromatography on DE 52 pH 8.5 and the subunits were recombined to produce cAMP dependent kinase activity. Kinase reaction mixture was added to the regulatory subunit preparation, then the reaction was initiated by adding the catalytic subunit, (i.e., there was no preincubation of the subunits before beginning the kinase reaction), and the reactions were allowed to proceed for various periods of time. As shown in Figure 10, even after

only one minute of incubation, the subunits had achieved a considerable degree of reconstitution, and the amount of reconstitution increased gradually between the one and ten minutes of incubation tested. While these experiments do not provide precise kinetic data on this process they both indicate that the subunits associate rapidly, within a matter of minutes. As shown in Figure 10, the subunits can actually recombine while the kinase reaction is in progress. In addition, since MgATP was present only in the reaction mixtures during these experiments, it is clear that a preincubation period in the presence of MgATP is not necessary to allow the subunits to associate.

3.3.3 Effect of NaCl and pH on reconstitution

We also examined the effects of pH and high NaCl concentrations on reconstitutions. The reassociation of mammalian type II subunits is blocked by 500 mM NaCl which is sometimes included in extraction buffers to permit determination of *in vivo* subunit levels (Corbin *et al.* 1973). The effects of high NaCl on the *Dictyostelium* kinase have been difficult to assess as NaCl as well as KCl are potent inhibitors of this enzyme (Fig. 11). This problem was somewhat alleviated by using very active enzyme samples which exhibited low but measurable activity even when significantly inhibited. When subunits were mixed together

at various salt concentrations to form holoenzyme the activity ratio was found to increase from 0.3 in the absence of NaCl to 0.75 in the presence of 500 mM NaCl (Fig. 12). If the total activity was unaffected by the salt, such a change in activity ratio would indicate inhibition of reassociation. However, since NaCl does inhibit activity, this change in activity ratio is probably at least partially due to loss of activity produced by the salt. In any event, some cAMPdPK activity was produced even during the high salt treatment, so reconstitution was not completely inhibited. As measured by activity ratio, reconstitution also occurred more readily at physiological pH values, (Fig. 12,13). However, the same problem exists in interpreting these results because low pH also depresses total kinase activity. These experiments demonstrate the necessity for exercising caution when interpreting results based upon activity ratios.

3.3.4 Additional properties related to MgATP

In addition to its effect on reassociation of the type I subunits, MgATP inhibits cAMP binding to the mammalian type I but not type II holoenzyme (Haddox et al. 1972, Beavo et al. 1974,1975). However as shown in Figure 14, there was no effect on the binding of ^{3}HcAMP to the Dictyostelium holoenzyme in the presence of 0.2 μM MgATP. Scatchard

analysis (Scatchard 1949) of the data reveals the Kd values for cAMP to be essentially equivalent in the presence and absence of MgATP (9.3 and 10.3 nM, respectively). We also assayed for MgATP binding activity in the holoenzyme samples. Although a low affinity (Kd ^{350} mθ┤ θλV)→ βινφινλ activity was present in the holoenzyme samples prepared through the steps of DE 52 and S300, the two activities did not co-purify on IEF (not shown).

3.4 DISCUSSION

We have investigated the conditions under which the Dictyostelium cAMPdPK will dissociate and thereby become active, and associate, leading to inactivation. In addition we sought to further compare the Dictyostelium enzyme to the mammalian cAMPdPK type I and type II prototype isozymes.

Analysis of holoenzyme dissociation by cAMP was performed by using density gradient centrifugation. We observed that inclusion of cAMP in the gradient would lead to a small but reproducible shift in the sedimentation behavior of the protein kinase and conclude that this represents dissociation of the holoenzyme into free catalytic subunit. By using ^{3}HcAMP to produce this effect we could observe the behavior of the regulatory subunit and found that it also sedimented as the free subunit.

Previous attempts in this laboratory to demonstrate cGMP- or cAMP-induced dissociation by chromatographic methods were for the most part unsuccessful (Rutherford et al. 1984). In fact dissociation was observed only when NaCl was included with the cGMP. In the present report NaCl was not required to produce dissociation during density gradient centrifugation, and inclusion of NaCl in these experiments did not affect the results. The requirement for NaCl and lack of dissociation seen in the earlier experiments may have been due to interfering protein-resin interactions which were circumvented by using the density gradient technique.

Histone was also tested for the ability to produce dissociation. We found that although histone VII-S was capable of activating the enzyme to a considerable extent in the absence of cAMP it did not appear to cause subunit dissociation during density gradient centrifugation. The recovery of cAMP-binding activity from these density gradients was very low, a factor which may be related to the mechanism of histone action. One possibility may be that histone is indeed promoting the release of the subunits from each other and reducing the cAMP binding capability, but the subunits then remain connected via the histone. The increase in the molecular weight of the holoenzyme produced

an attached histone (ca. 14,000 daltons) might not be enough to appreciably alter the sedimentation properties of the enzyme under our conditions. Certainly however, this idea is speculative and further experiments will be required to elucidate the mechanism of the histone effect. It also remains tempting to speculate on the possible role that phosphorylation of Dictyostelium histones by this enzyme might play in development.

In the density gradients holoenzyme activity regularly sedimented near the BSA marker while the catalytic and regulatory subunits sedimented slightly above ovalbumin. This would indicate subunit molecular weights in close agreement with previously determined values (catalytic subunit Mr=40,000; regulatory subunit Mr=41,000), (Schoen et al. 1983, Majerfeld et al. 1984, deGunzburg et al 1984), and a dimeric holoenzyme structure. DeGunzburg et al (1984) have proposed dimeric holoenzyme structure based on density gradient analysis of reconstituted holoenzyme and gel filtration analysis of native enzyme. However, gel filtration data from our laboratory (Rutherford et al. 1982) and others (Majerfeld et al. 1984) is more indicative of a tetrameric structure. while others (Schoen et al. 1983) have suggested an octameric structure. None of the variations in the density gradient procedure that were tried resulted in

holoenzyme activity sedimenting to a position corresponding to a tetramer, although higher speeds and longer runs tended to result in loss of cAMP dependency and recovery of kinase activity at the same position as catalytic subunit. The nature of this technique may thus result in holoenzyme activity being reduced to its smallest possible form, i.e., a dimer. It is also conceivable that this enzyme can exist at least *in vitro* in various multimeric forms. Further studies will be necessary to clarify this issue.

The reassociation behavior of the subunits was investigated by either dissociating the holoenzyme with cAMP and then removing the cAMP or by recombining separated subunits to produce holoenzyme activity. We investigated the effects of time, pH, NaCl, and MgATP on reconstitution.

Low pH and high salt resulted in higher activity ratios which may indicate inhibition of subunit reassociation. However, since these treatments affected the overall kinase activity it is not possible to determine how much of the change in activity ratio is due to inhibition of reassociation and how much is due to enzyme inhibition. Further experimentation would be necessary to distinguish between these two alternatives. Analysis of these effects using the density gradient technique would be illuminating but would be difficult to perform because of the amount of

inhibition produced by these treatments. Nevertheless the experiments shown in this report did demonstrate that at at least some degree of reconstitution occurred under the conditions of low pH and high salt, i.e., there was not a total inhibition of reassociation. This NaCl effect thus appears to be different from both the mammalian type II in which reconstitution is inhibited by high salt, and type I, in which reconstitution is unaffected by high salt. The pH result is also slightly different from that reported by deGunzburg et al (1984) who found complete inhibition of reconstitution below pH 6.

The two methods used to measure the rate of subunit reassociation gave similar results. Reassociation occurred rapidly and did not require a preincubation with or without MgATP. Rapid reassociation would presumably allow for fine control over the activity of this enzyme in vivo under conditions when cAMP was degraded or sequestered within the cell. Since a cAMP phosphodiesterase is active in Dictyostelium stalk cells (Brown and Rutherford 1980) the possibility exists for such a control system to be utilized.

Mammalian cells contain two cAMPdPK isozymes, type I and type II, which exhibit many different properties (Hofmann et al. 1973, Beavo et al. 1974,1975, Corbin et al 1975). The physiological significance of these two isozyme

classes is as yet unclear although it has been noted that the type I isozyme tends to be the predominant form during proliferative events while high PK II levels tend to be associated with differentiation processes (Russell 1978, Handschin and Eppenberger 1979). While all mammalian cells tested so far contain both isozymes, there is no evidence for isoenzymes in Dictyostelium. Because the isozymes may have different physiological roles it is clearly of interest to compare and contrast the Dictyostelium enzyme with the mammalian counterparts. The most commonly used criteria for distinguishing the cAMPdPK isozymes are the molecular weight differences between the regulatory subunits, and differential elution of the holoenzymes from DEAE resin. Since the Mr of the Dictyostelium regulatory subunit is considerably smaller than either of the mammalian prototypes this is not a useful characteristic for comparison. The Dictyostelium cAMPdPK can be eluted at low ionic stength from DEAE although the subunits tend to dissociate when linear salt gradients are used. However, the large difference in regulatory subunit Mr probably also affects the DEAE elution profile making this, too, a less than ideal characteristic for comparison.

However, there are other less commonly used characteristics that can be used for this purpose (see Table

3). For instance, there are several properties related to MgATP which clearly distinguish between mammalian type I and type II. PK I contains a high affinity MgATP binding site, MgATP inhibits cAMP binding to PKI, and reconstitution of the type I subunits is slow or absent in the absence of MgATP. Although Dictyostelium holoenzyme samples contained a low affinity MgATP binding activity this did not co-purify with the holoenzyme through IEF. MgATP did not decrease cAMP binding to holoenzyme and the Dictyostelium subunits reassociated readily in the absence of MgATP. In all of these respects the Dictyostelium enzyme is more similar to the type II isozyme.

Other ways in which the Dictyostelium enzyme resembles PKII are in its elution properties from cAMP agarose (Dills et al, 1979, Rutherford et al. 1984) and ability to be autophosphorylated (Majerfeld et al. 1984). However, the nucleotide specificity of the Dictyostelium enzyme is similar to PKI (De Witt et al. 1982), and the Dictyostelium enzyme is intermediate or different from both types with respect to salt and substrate effects on association and dissociation properties (Table 3).

Clearly then, the Dictyostelium enzyme does not fit easily into either category although it strongly resembles type II in two properties highly specific for PKII -

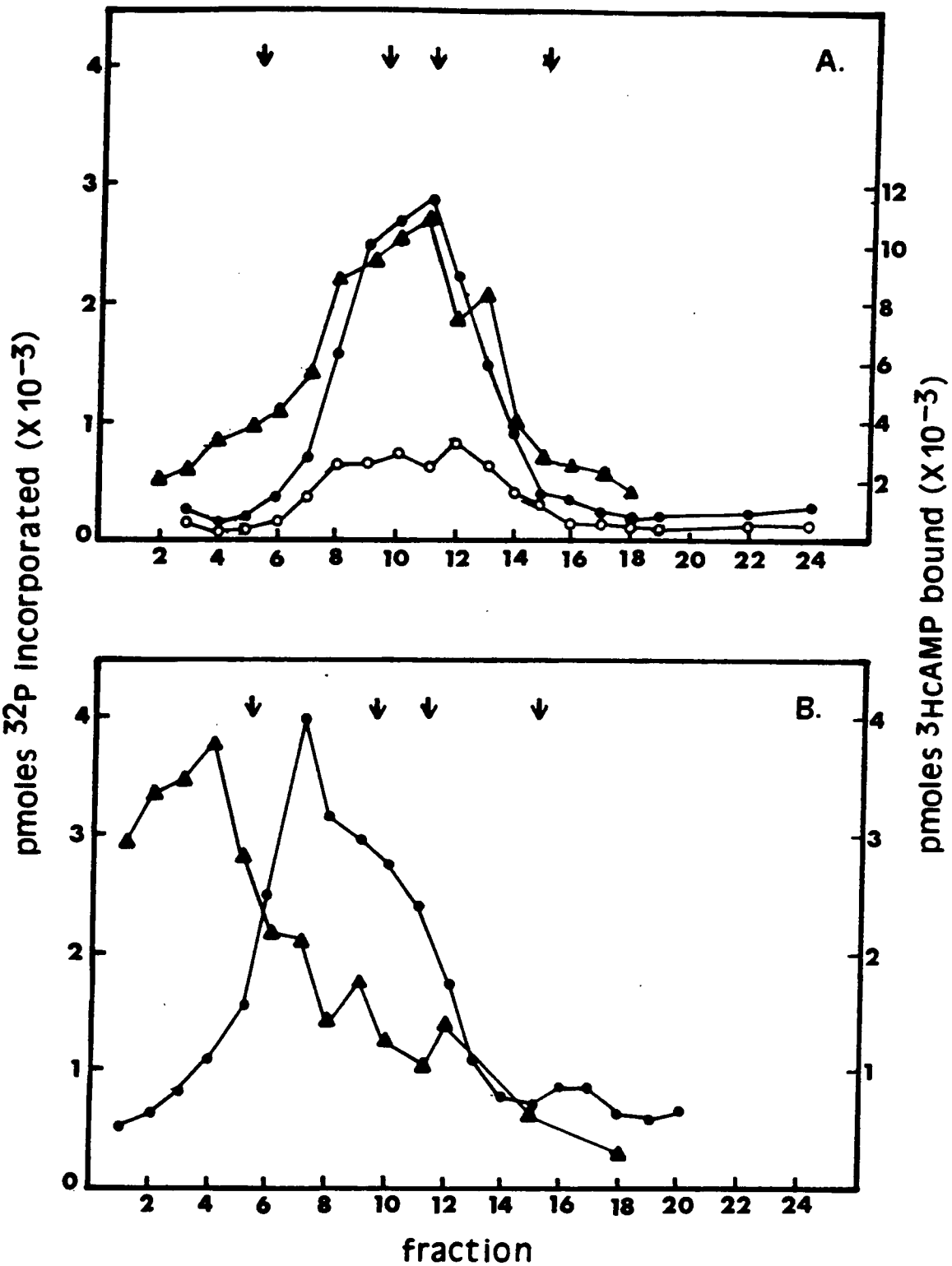
autophosphorylation and MgATP effects. It is interesting to note that in the lower eukaryotes Neurospora (Glikin et al. 1982), Blastocladiella (Brochetto-Braga et al. 1984), and Mucor (Moreno and Passeron 1982) which have life cycles in which cAMP plays an important although as yet undefined role, only the type II kinase has been isolated. In light of these facts and because of the presumed role of cAMP in differentiation of the cell types in Dictyostelium, it is tempting to label the Dictyostelium enzyme a type II kinase. However, this enzyme is different from PKII in enough respects to make such a classification questionable, and categorization per se of the Dictyotelium enzyme as type I or II may not be possible. Perhaps however, knowledge of its special properties may still provide a basis for comparison with mammalian isoenzyme function as these roles become increasingly understood.

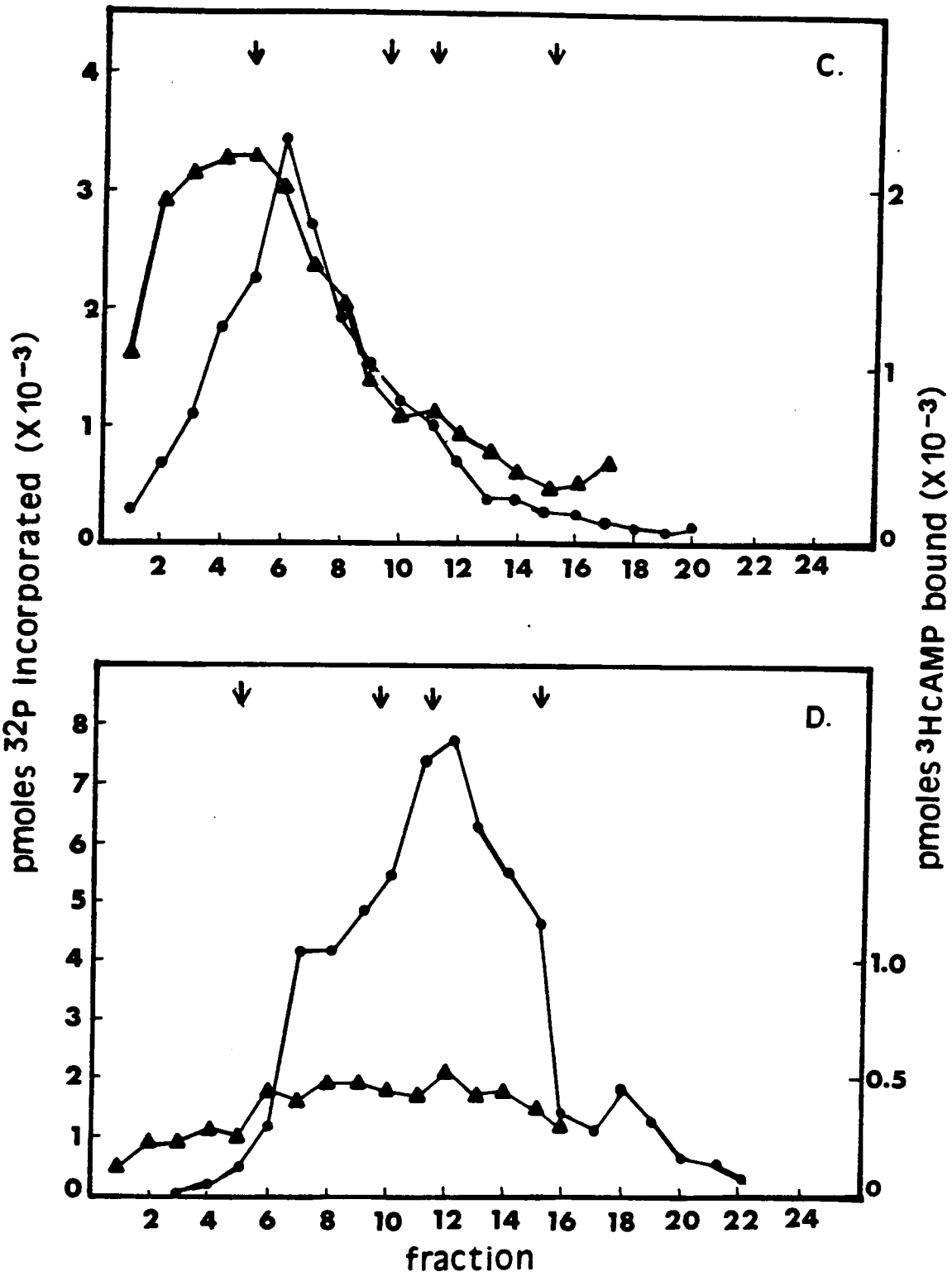
It is hoped that this study, by increasing our knowledge of factors which may be of physiological relevance in the control of this kinase, may help expand our undertanding of cAMP action in Dictyostelium. Although considerable information has now been accumulated by this laboratory and others on the biochemical properties of the cAMPdPK, its role in mediating cAMP related developmental events is still unknown. Futher work in this laboratory

will be aimed at elucidating the cell-specific activities of the enzyme and investigation of the enzyme's endogenous substrates.

Figure 7. Density gradient centrifugation of cAMPdPK and subunits. Fractions were collected from the tops of the gradients. Protein standards (arrows from left to right) were lysozyme (Mr = 19,000) ovalbumin (Mr = 43,000), bovine serum (Mr = 67,000), and gamma-globulin (Mr = 150,000). The arrows indicate the average positions of the standards from all experiments. The range of variation in sedimentation of the standards was ±0.5 fractions. In all experiments kinase activity was assayed in the presence and absence of cAMP. When the activity was the same the results were averaged.

A. Holoenzyme purified through S300. Kinase activity in the presence (●) and absence (○) of cAMP; cAMP binding activity (▲).
B. Free catalytic (●) and free regulatory (▲) subunits prepared from DE52 chromatography.
C. Protein kinase (●) and cAMP binding activity (▲) recovered from a holoenzyme sample sedimented through a density gradient which contained 125 nM ^{3}HcAMP.
D. Protein kinase (●) and cAMP binding activity (▲) recovered from a holoenzyme sample sedimented through a gradient which contained 0.4 mg/ml histone VII-S.


A.
B.
pmoles 32P incorporated (X10-3)
pmoles 3HcAMP bound (X10-3)
fraction
0
1
2
3
4
2
4
6
8
10
12
14
16
18
20
22
24


C.
D.
pmoles 32P incorporated (X10-3)
pmoles 3HcAMP bound (X10-3)
fraction
0
1
2
3
4
5
6
7
8
0.5
1.0
2
4
6
8
10
12
14
16
18
20
22
24

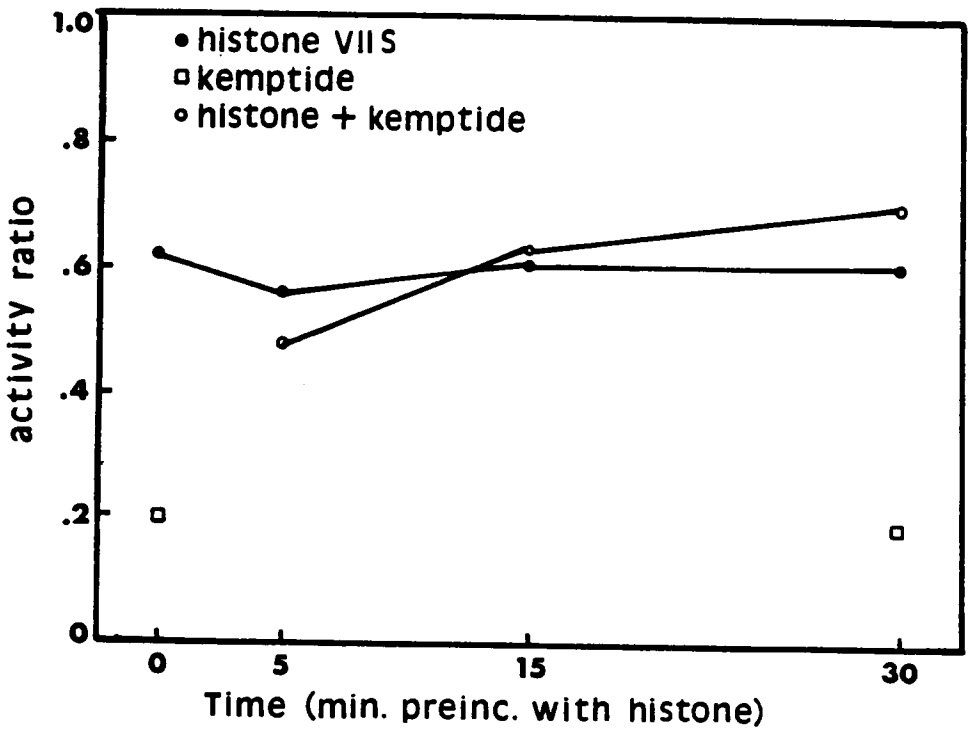


Figure 8. Effect of substrate on activity ratio. Holoenzyme was assayed in the presence and absence of cAMP with histone alone (●), Kemptide alone (□), or histone and Kemptide together (○). The preincubations were performed in the presence of histone and the reactions were initiated by adding ATP (and Kemptide in the combination assays). Histone concentration was 0.4 mg/ml, Kemptide was 35 μM. Each point is the average of duplicates which varied less than ten percent.

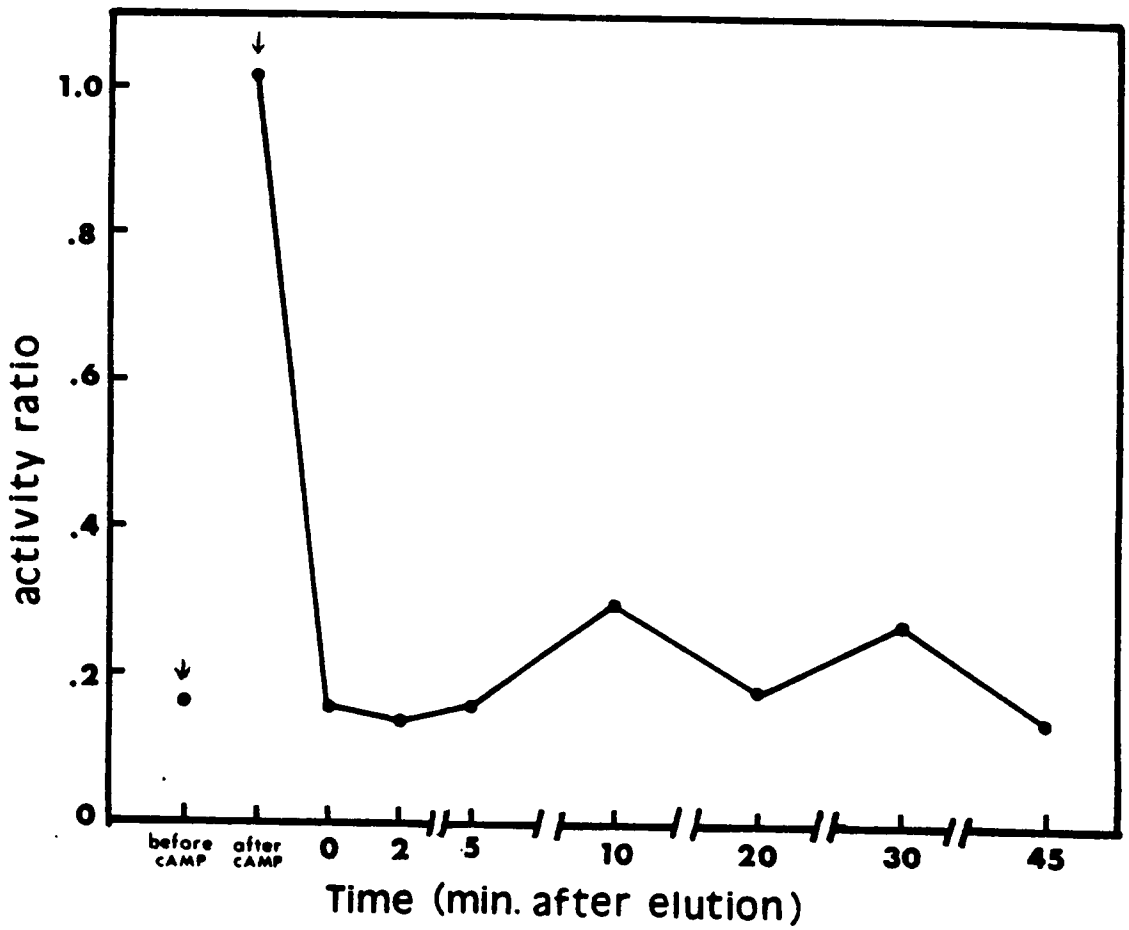


Figure 9. Time course of reconstitution after removal of cAMP. Activity ratios of holoenzyme before and after addition of cAMP to sample (arrows), and at various time intervals after removal of the cAMP by passage of the sample over a PD10 column. About 2 minutes elapsed between loading the sample onto the column and its elution, and another 2 minutes elapsed during the reaction period. Each point is the average of duplicates.

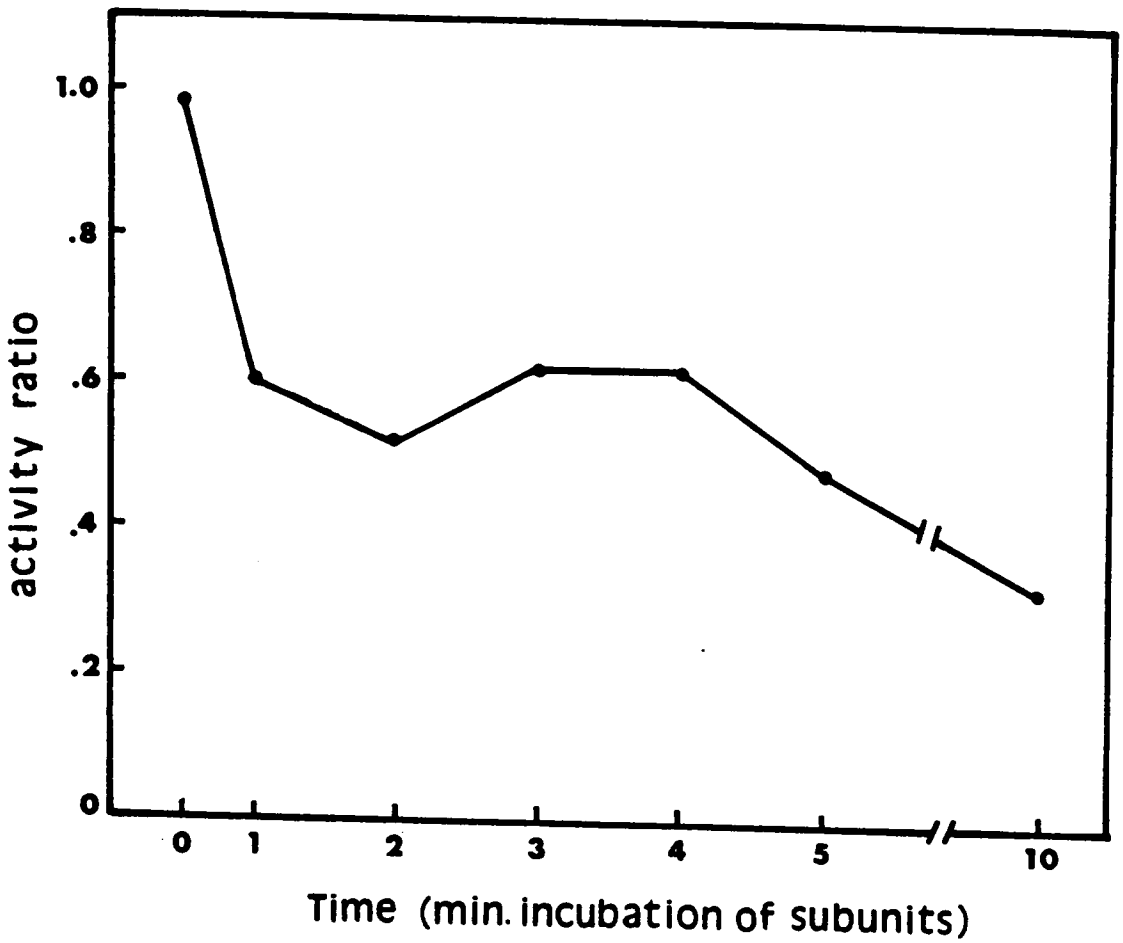


Figure 10. Time course of reconstitution of dissociated subunits. Activity ratios of reconstituted holoenzyme. Catalytic and regulatory subunits were prepared from DE52 as described in methods. Protein kinase reaction mixture was added to aliquots of regulatory subunit, and reactions were initiated by adding aliquots of catalytic subunit. Reactions were allowed to procede for the time intervals indicated. Each point is the average of duplicates.

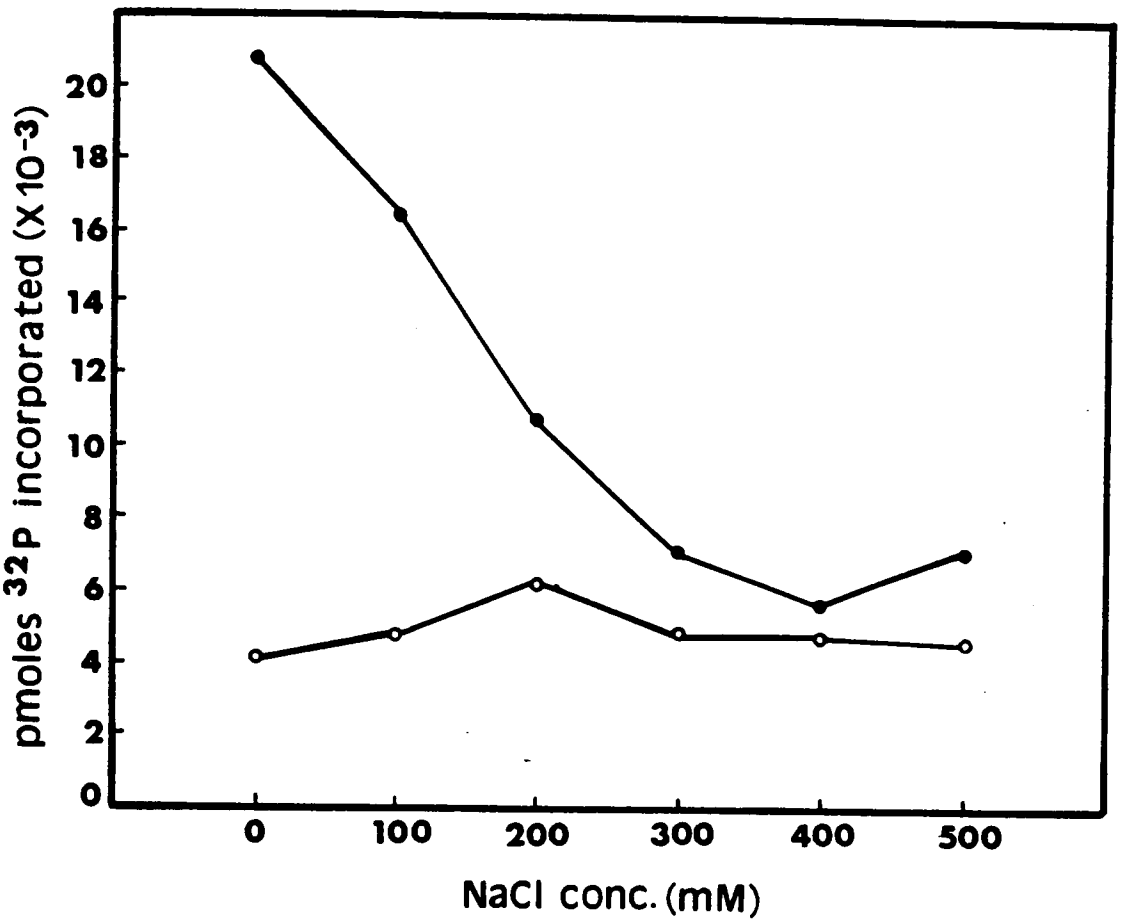


Figure 11. Effect of NaCl on reconstitution of holoenzyme. Catalytic and regulatory subunits were prepared from IEF. Aliquots of each were mixed in the presence of various amounts of NaCl, preincubated for 15 minutes, then assayed for protein kinase activity in the presence (●) and absence (O) of cAMP. The values shown are the averages of triplicate determinations.

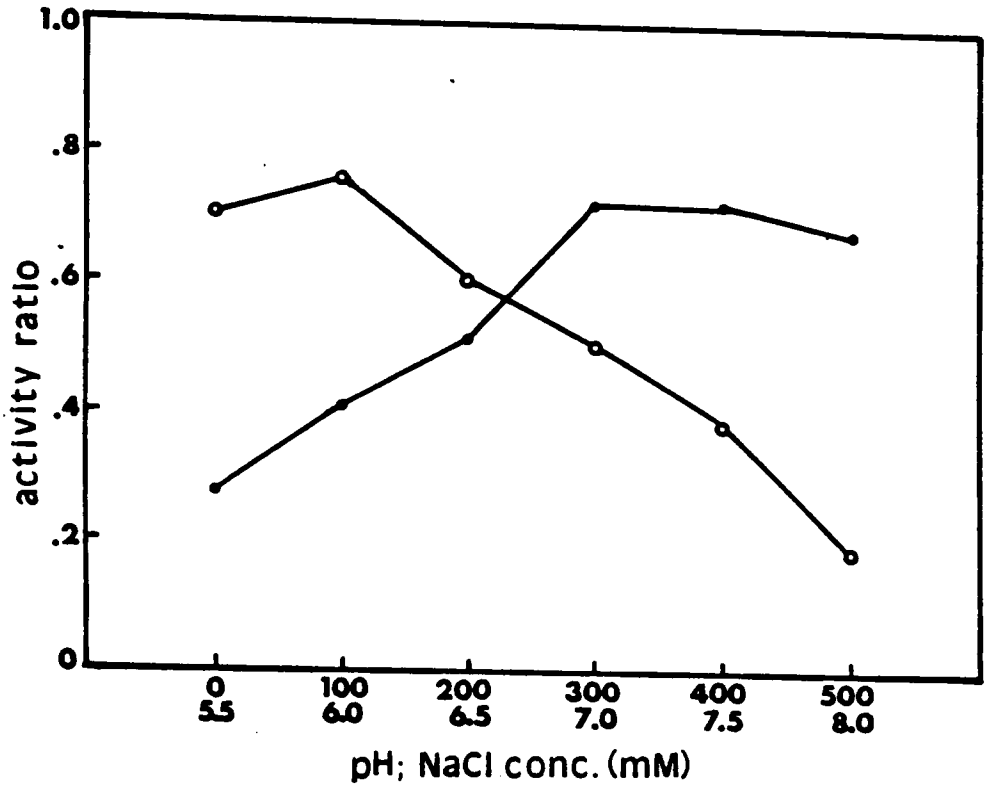


Figure 12. Activity ratios of reconstituted holoenzyme in the presence of NaCl (●) and at various pH values (○). These values are the combined results of three replicates of the NaCl experiment shown in Figure 11 and two replicates of the pH experiment shown in Figure 12.

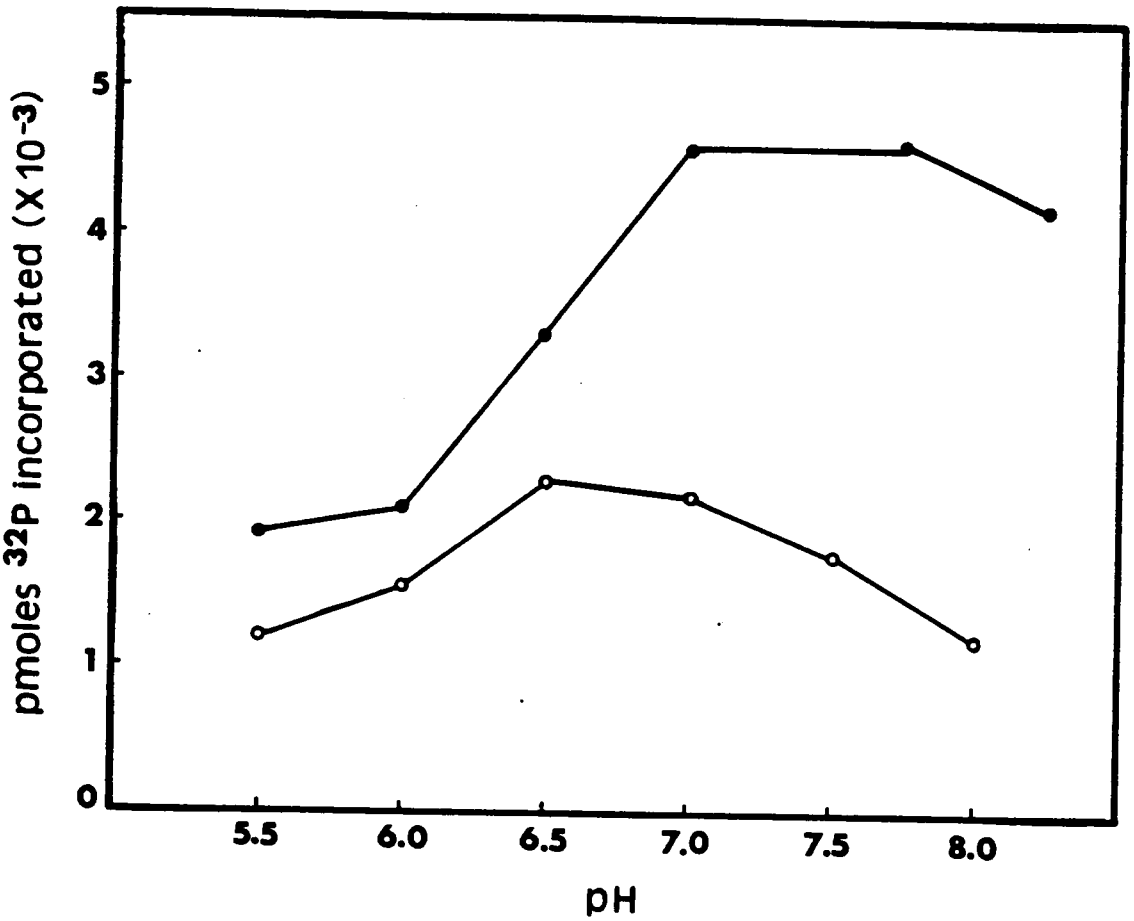


Figure 13. Effect of pH on reconstitution. Catalytic and regulatory subunits prepared from IEF were dialyzed into 5 mM TAMB pH 7.0. Aliquots of each were then mixed and assayed for protein kinase in the presence (●) and absence (O) of cAMP using reaction mixtures made with 50 mM buffers at each indicated pH. Phosphate buffer was used for pH 5.5-6.5, Tris buffer for pH 7.0-8.0. The values shown are the averages of triplicate determinations.

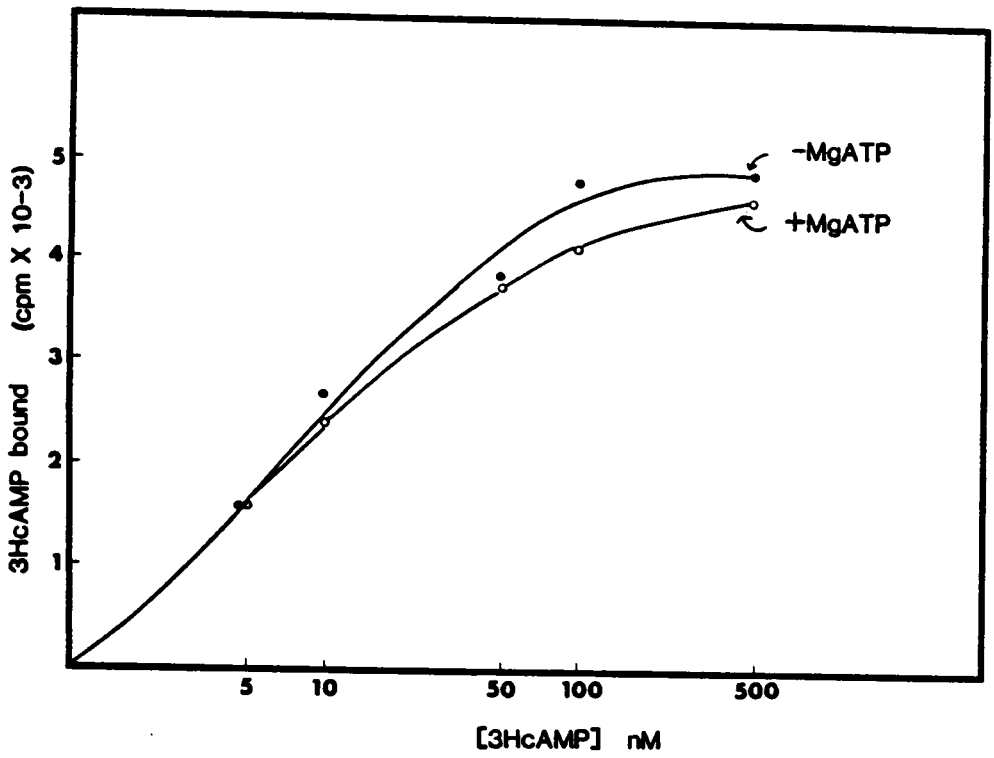


Figure 14. Effect of MgATP on 3HcAMP binding to holoenzyme. Identical S300 holoenzyme samples were assayed for ^{3}HcAMP binding at the indicated final concentrations of the nucleotide in the presence (O) and absence (●) of 200 µM ATP and 2 mM MgCl2. Samples were preincubated with MgATP for 15 minutes before the addition of reaction mixture, then incubated with the reaction mixture for 15 minutes before filtering. Each point represents the average of triplicate determinations.

Table 3. Comparison of Dictyostelium and mammalian isoenzymes.[1]

Property	Type I	Type II	Dictyostelium
elution from DEAE	<100 mM NaCl	>100 mM NaCl	<100 mM NaCl
Mr holo reg cat	170,000 48,000 40,000	190,000 54,000 40,000	170-190,000/70,000 41,000 40,000
dissociated by histone	fast-prevented by MgATP	no	fast activation in presence of MgATP, but no dissociation
reassoc. in 0.5M NaCl	yes	no	slight amount
MgATP effects a)reassoc. after removal of cAMP	slow(-MgATP) fast(+MgATP)	fast(+/-MgATP)	fast(-MgATP)
b)high affinity MgATP binding site.	yes	no	no
c)MgATP inhibits cAMP binding	yes	no	no
elution from cAMP agarose	30 mM cAMP, 30oC, 60 min.	1 mM cAMP, 4oC, 15 min.	1 mM cAMP, 4oC, 30 min.
R autophosphorylated	no	yes	yes

[1] References for the data are given in the text.

Chapter 4
Localization of cAMP-dependent protein kinase in *Dictyostelium discoideum*.

4.1 INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is an excellent organism to utilize for the study of developmental processes. Upon starvation a population of identical unicellular amoebae enters a sequence of events resulting in the formation of a fruiting body composed of a mass of spore cells supported by a stalk (Loomis 1982). The process of aggregation is initiated by the secretion of adenosine 3'5' cyclic monophosphate (cAMP) from some of the cells (Konijn *et al*. 1968). In addition to its role in chemotaxis cAMP has been implicated as a regulatory molecule in later stages of development. For instance, cAMP accumulates during the culmination stage of development (Brenner 1978, Pahlic and Rutherford 1979, Abe and Yanagisawa 1983) and becomes localized in specific cell types of the culminate (Pan *et al*. 1974, Brenner 1977, Merkle *et al*. 1984). Cyclic AMP is required for both stalk cell (Bonner 1970, Gross *et al*. 1978) and spore (Town *et al*. 1976, Kay *et al*. 1978) differentiation. Stalk cells appear to be chemotactically attracted to a cAMP source (Maeda and Maeda 1974, Sternfeld

and David 1981, Matsukuma and Durston 1979). Exogenously applied cAMP causes the premature induction of several developmentally regulated enzymes (Gerisch et al 1975, Darmon et al 1975) and may induce and maintain postaggregation gene expression (Kay 1979, Landfear et al. 1982). However, the specific metabolic processes actually controlled by cAMP and the mechanism by which these effects are mediated are as yet unknown. Because the only known mediators of cAMP action in eukaryotic cells are cAMP dependent protein kinases (cAMPdPKs), it is likely that the cAMP-induced developmental events in Dictyostelium are mediated by this enzyme. This laboratory (Rutherford et al. 1982) and others (deGunzburg and Veron 1982, Cooper et al. 1983, Majerfeld et al. 1984, Schoen et al 1984) have isolated and partially characterized a cAMPdPK from Dictyostelium.

Previous work from this laboratory has shown a striking localization in Dictyostelium of the enzymes which regulate cAMP levels, adenylate cyclase and cAMP phosphodiesterase. The cAMP synthetic enzyme adenylate cyclase is present in much higher levels in the prespore mass of culminating individuals than in the stalk cells (Merkle and Rutherford 1984). Conversely, the activity of the degradative enzyme, cAMP phosphodiesterase (PDE) is low in the prespore mass but

increases greatly in the prestalk cells (Brown and Rutherford 1980). As expected from this enzyme distribution cAMP itself is present in a gradient in culminating individuals (Merkle et al. 1984).

In view of the localization of these enzymes and cAMP it was of interest to investigate the distribution of the cAMPdPK. If the kinase is mediating a cAMP signal related to cell-type differentiation it is conceivable that the kinase is also cell-type specific. This hypothesis is supported by the recent report of 5-fold higher amounts of the cAMPdPK subunits in the prespore than in the prestalk cells of pseudoplasmodia when the cells had been separated on density gradients of Percoll (Schaller et al. 1984). In this paper we report on the distribution of the Dictyostelium cAMPdPK at several stages of development in tissue pieces separated by microdissection.

4.2 MATERIALS AND METHODS

4.2.1 Growth and Differentiation of Cells

Amoebae of Dictyostelium discoideum (wild type NC-4) were grown from spores in association with Escherichia coli on nutrient agar at 23°C as described by Rutherford (1976). The amoebae were harvested after 48h of growth at 23°C then

washed three times (18000 x g for 1 minute) with ice-cold salt solution (Bonner, 1947) to remove the bacteria.

For the study of individuals, the amoebae were harvested and washed as above, but were plated on Whatman Number 50 filter paper that was overlaid on non-nutrient agar (Rutherford and Harris, 1976), and allowed to differentiate at 19°C. At various stages of development the organisms were frozen quickly by removing the filters to a metal surface at -70°C. The individuals were then lyophilized while still on the filters, and stored under vacuum at -70°C.

4.2.2 Description of Microtechnique

On the day of the assay vials were brought to room temperature. Individuals at the desired stage of development were selected under a dissecting microscope and removed from the vials. Sections were cut from the individual by dissection using a microscalpel composed of small section of razor blade attached to a copper wire embedded in a dowel (Rutherford 1976). The sections, weighing from 50 ng, were transported by hair tip. The weight was measured on a quartz-fiber microbalance as described by Lowry and Passonneau (1972). Sections were cut as closely to the same size as possible to avoid artifactual differences in enzyme activity levels due to the amount of

tissue used. Careful drawings were made at the time of dissection in order to express activity with relation to the location of the section within the individual.

4.2.3 Microassay of Protein Kinase

Freeze-dried tissue sections, cut and weighed on the day of the assay, were placed in the bottom of 100 µl microtiter wells. Protein kinase activity was assayed by adding a volume of 28.7 µl reaction mixture to each well. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 3 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM benzamidine, 50 mM phenyl phosphate, 35 µM Kemptide, 15 µM ATP, and 0.4-2.0 µM [γ-^{32}P]ATP (0.4 Ci/mmole), with or without 20 µM cAMP. After addition of reaction mixture the wells were covered with a microscope slide to reduce evaporation. After a 2 h incubation at room temperature the reaction volumes were removed to 1 cm square pieces of P81 filter paper. The papers were carried through seven acetic acid washes, 15 min each (the first two 30%, the remainder 15%), dehydrated in acetone, and dried for determination of radioactivity. Protein kinase activity in the sections was quantified on the basis of section weight and is expressed as femtomoles phosphate incorporated into Kemptide per minute per microgram dry weight.

4.3 RESULTS

4.3.1 Validation of the assay

Figure 15 shows the linearity of the kinase reaction with time. Because of the small size of the tissue pieces it was desirable to incubate the reactions as long as possible to increase the assay sensitivity. Under the conditions described in methods the rections could be incubated up to two hours with good linearity and the ratio of activity in the presence and absence of cAMP was constant during the two hours. Incubation of the reaction for more than two hours resulted in a decrease in linearity (not shown). Less than 25% of the substrates were utilized in these assays.

We had previously detected in batch-prepared extracts a casein kinase activity which is distinct from the cAMPdPK. The activity of the casein kinase is not affected by cAMP and does not phosphorylate Kemptide. However, in order to clearly substantiate the identity of the kinase detected in the microassay Dictyostelium individuals were also assayed with casein as the substrate and without any exogenous substrate. No phosphorylation activity was detected in either of these cases (not shown). Therefore it appears that only cAMP-dependent Kemptide kinase activity is being assayed in these experiments.

The linearity of the Kemptide kinase reaction with respect to tissue weight is shown in Figure 16. Linearity actually extended to pieces approximately twice as large as those shown (i.e., between 2 and 3 µg). However for most of the experiments reported here the tissue pieces were kept within the range shown, between 0.2 and 1.0 µg. When assaying for cAMPdPK in batch preparations as described in Rutherford et al. (1982,1984), very little to no holoenzyme activity could be detected in crude sonicates or in 100,000 x g supernatants. We had observed, however, that by diluting these preparations 20-100 fold we could often detect holoenzyme activity. Likewise it was found in this study that little cAMPdPK activity was detectable in whole individuals or pieces of tissue were incubated in small reaction volumes (1-5 µl), but that increasing activity was obtained when larger reaction volumes were used. This is probably due, at least in part, to diluting-out of a Kemptide phosphatase. Because of this active phosphatase and/or other factors, linearity of the reaction at this reaction volume was reduced with larger pieces (above 3 µg). The sensitivity of the assay with smaller pieces extended to weights of approximately 0.1 µg. Within each experiment tissue pieces were kept as close in size as possible to help reduce artifacts due to size variation. Nevertheless, there

was a certain amount of variability observed even in pieces which were of similar size.

4.3.2 Solubilization of the enzyme

Demonstration of the solubility of the enzyme was performed in the following manner. Reaction mixture containing cAMP was added to a tissue section in the bottom of a microtiter well and was stirred gently by blowing through the constriction pipette used to transfer the reaction mixture. After ten minutes half of the volume was transferred to an adjacent well and the reactions were continued for the remainder of the two hours. Each volume was then pipetted to separate pieces of P81 paper and treated as described in methods. Because essentially equal amounts of phosphorylated product were present in each of the paired wells (Fig. 17), the kinase activity must have become solubilized during the first ten minutes after the addition of the reaction mixture and half of the enzyme transferred to the second well. This finding is consistent with our earlier results showing the kinase present only in supernatants of sonicated cells and not in the pelleted portion of the centrifuged material. We had hoped to be able to use this method to assay the same pieces of tissue in the presence and absence of cAMP by first adding reaction mixture which did not contain cAMP, removing half the volume

to the adjacent well and then adding cAMP to the second half. This method did work well in some instances but it was not adequately reproducible and did not result in the detection of as much cAMP dependency as assaying paired sections of tissue as described below.

4.3.3 Activity of enzyme throughout development

Although we had previously determined the activity of the cAMPdPK at three different stages of devlopment by harvesting cells from batch preparations on agar (Rutherford et al. 1982), it was possible to perform a more precise determination of the activity of this enzyme in individuals at each stage uncontaminated by asynchronous cells (Fig. 18). To perform this study, individuals at each stage of development were bisected through the long axis. One half of each was assayed for Kemptide kinase in the presence of cAMP, the other half was assayed in the absence of cAMP. The results show cAMP-dependent activity even in aggregates before the formation of tips, considerably earlier than we had detected it in batch preparations. Kinase activity increased after aggregation, and reached a peak during the pseudoplasmodium and culmination stage. The activity decreased during sorocarp construction although it remained quite detectable and cAMP dependent. A low but detectable amount of non-cAMP dependent Kemptide phosphorylation was

observed in amoebae at 0 h of development and a higher amount of non-cAMP dependent activity was seen in streaming aggregates. Whether this actually represents low amounts of catalytic subunit or whether it is a different kinase which can phosphorylate Kemptide was not determined.

4.3.4 Distribution within pseudoplasmodia

The studies of Schaller et al (1984) who separated slug-stage prespore from prestalk cells by using Percoll density gradients showed that the subunits of the cAMPdPK were present in 5-fold higher amounts in the prespore cells than in the prestalk cells. However, this finding was not substantiated by the results of this microdissection study. A total of nine slugs were dissected perpendicularly to the long axis into 4,5,6, or 11 pieces, depending on the length of the slug, and assayed for kinase as described. Although there was a certain amount of variability observed between individual pieces, in no case was there a 5-fold difference in activity seen between front and rear sections (Fig. 19). Even when individual papilla were dissected apart from the rest of the sections the activity in those pieces was equivalent to the activity in the prespore sections. Although we did not utilize the same control experiments as in Schaller et al. (1984), i.e., assay of UDPG polysaccharide transferase or labelling of cells with

prespore antibody, we have dissected the slugs in these experiments into as many as eleven pieces. Certainly if the forward two-fifths to one-third of slugs are the prestalk cells (Raper 1940) we would have separated prestalk from prespore in some of these sections.

In some of these experiments slug sections were assayed entirely in the presence of cAMP. We also performed some of the assays in the presence and absence of cAMP to determine if there was any pattern seen in cAMP dependency, i.e., was all of the kinase present as holoenzyme or was free catalytic subunit present in some of the cells? To perform these experiments slugs were dissected across the long axis as described above, then each piece was bisected through the long axis to produce matching sections. One piece of each pair was then assayed in the presence of cAMP, the other piece in the absence of cAMP. As shown in Figure 19, cAMP-dependent kinase activity was observed in every front-to-back section, i.e., there was no evidence of free catalytic subunit in any section of the slug. Bisection of papilla for assay by this method resulted in pieces too small to be asayed with linearity. Therefore several papilla were assayed individually either in the presence (n=15) or absence (n=15) of cAMP. Since the average activity of these groups was equivalent to the activity of the other slug

sections, we conclude that the kinase activity in the tips is also cAMP dependent.

4.3.5 Distribution in Culminating Individuals

Previous work from this laboratory on localization of cAMP and its associated enzymes showed the localization occurring predominantly during the later stages of development, culmination and sorocarp construction. Culmination was also the stage where the most localization of the cAMPdPK was detected. As shown in Figure 20, there was no detectable change in kinase activity from the base to the apex of the prespore mass, and assaying matched tissue sections as described for pseudoplasmodia showed that the kinase was holoenzyme in all pieces. The tips were assayed in groups using the method described for tips of pseudoplasmodia. A total of 18 tips were assayed in the presence of cAMP and 15 tips were assayed in the absence of cAMP. The activity in the tips as determined by this method was not significantly different from the activity of the prespore cells. Since the stalks were much too small to be bisected and assayed in matching pairs they were also assayed in groups. Stalks from very large individuals could be assayed alone; in most cases, however, it was necessary to pool 2-4 stalks to accumulate a dry weight of above 0.2 µg in order to be within the linear assay range for tissue.

A total of 45 determinations (consisting of the assay of one stalk or 2-4 pooled stalks) was performed. Twenty-five of the determinations were performed in the presence of cAMP, twenty were performed in the absence of cAMP. As shown in Figure 20, the kinase activity activity was considerably lower in the stalks than it was in the prespore mass, but it was still cAMP-dependent. In some experiments large stalks were cut in halves (tops and bottoms) and assayed for kinase activity in the presence of cAMP. In a total of 5 cases there was no difference in activity between the apical and basal halves.

4.3.6 Distribution in Sorocarps

Prespore or spore masses of early sorocarps were bisected and each half was dissected from apex to base. Paired sections were then assayed in the presence or absence of cAMP. As shown in Figure 21, the kinase activity is still cAMP dependent, although lower than that seen during culmination. There was no difference in activity seen from the apex to the base of the prespore mass. The kinase activity in the stalks of early fruits was determined by the same method used to assay stalks of culminating individuals. The overall activity was equivalent to that in the prespores mass and equally cAMP-dependent.

4.4 DISCUSSION

We have utilized microdissection and microassay techniques to determine levels of cAMP dependent kinase activity throughout development and in each cell type of Dictyostelium discoideum individuals. By using the methods described in this paper the kinase can be assayed with more sensitivity than it can with the assay of batch-prepared extracts as described in Rutherford et al. (1982). Thus we were able to detect both kinase and cAMP dependency in cells at very early stages (Fig. 18). Kinase activity was very low in amoebae and higher in streaming aggregates but was not activated by cAMP at these stages. After the formation of tight cell contacts later in aggregation the kinase activity increased and became cAMP-dependent. The maximum activity and cAMP-dependency occurred during culmination, the stage when cAMP levels are also maximum in Dictyostelium. The level of activity decreased somewhat during fruiting body construction. These experiments, in conjunction with others from this lab (Brown and Rutherford 1980, Merkle and Rutherford 1984, Merkle et al. 1984, Frame and Rutherford 1984), continue to provide support for the hypothesis that cAMP is involved with developmental events during the later stages of Dictyostelium differentiation.

Numerous experiments have been described which suggest a role for cAMP in cell-type differentiation in Dictyostelium although the nature of this role is still unknown. It seems logical, although still unproven, that the cAMPdPK described in this report and others (Rutherford et al 1982,1984) may be the mediator of these cAMP related developmental effects. Because the enzymes which regulate cAMP levels (Brown and Rutherford 1980, Merkle and Rutherford 1984) as well as cAMP (Merkle et al 1984) are highly localized during the later stages of development it was of interest to also determine the distribution of the cAMPdPK.

In pseudoplasmodia there were no detectable differences between any of the sections dissected from front to back, even when slugs were cut into as many as eleven pieces. It was also found by bisecting such pieces that the kinase activity was cAMP dependent throughout the slugs, i.e., there was no evidence of free catalytic subunits in any of the sections. This result is different from the report of Schaller et al. (1984) who detected 5-fold less kinase activity in the prestalk cells of pseudoplasmodia. It might be argued that no differences between front and rear sections were observed because all of the sections in this study displayed only the lower "pre-stalk" kinase activity

of Schaller et al. Although it is rather difficult to compare the results of studies performed with different techniques, the kinase activity we describe has the same approximate numerical value as that reported for the prespore cells by Schaller et al. (1984), but it is expressed as activity per µg dry weight rather than activity per µg protein. Correction of our data for this factor would increase the numerical value of kinase activity, therefore that we observed equivalent levels in anterior and posterior sections was not because of some failure to observe an activity as high as that seen in the prespore cells by Schaller et al. (1984). The detection of kinase activity in crude or partially purified samples can be hampered by the presence of phosphatases. The higher numerical value for kinase activity that we report may be a reflection of a higher dilution of phosphatase activity as compared to Schaller et al. Even under the conditions of our assay described here the phosphatases are not totally inhibited and are probably still reducing the amount of phosphorylated product that can be detected.

In culminating individuals the kinase activity is constant in sections assayed from the tip to the base of the prespore mass and is also cAMP-dependent in each of these regions. However there is a considerable drop in activity

between the tip region and the stalk. Unfortunately this assay is not sensitive enough to measure activity in very small sections of stalk, so that a very precise localization of where the activity begins to decrease could not be made. However, there was no difference between the activity measured in the apical and basal halves of the stalks.

Holoenzyme activity was also detected in both cell types during fruiting body construction. Compared to the stalks of culminating individuals the stalks of fruits had equivalent kinase activity. The activity in the prespore or spore mass was decreased compared to culmination, however, resulting in equal kinase activity in both spore and stalk cells of early fruiting bodies.

A possible mechanism by which kinase activity might be reduced in stalk cells during culmination would be the presence of an inhibitor. We have assayed in batch extracts for the presence of a heat-stable inhibitor of the kind present in mammalian cells (Walsh et al. 1971) but have seen no evidence for such a molecule. There was no detectable difference in activity between prespore sections of culminates which had an attached stalk and prespore sections which did not include a stalk, and addition of a boiled stalk extract to prespore pieces did not result in a significant decrease of kinase activity (data not shown).

This result does not preclude the possibility that an inhibitor is present in stalks but only in amounts adequate to inhibit a small amount of kinase activity. In any event, kinase activity is still detectable in the stalks during culmination even though it is lower than in the prespore cells.

It might also be argued that the amount of kinase activity detected in the stalk cells was artifactually low due to degradation of cAMP by phophodiesterase. However, the activity of PDE in the stalks (Brown and Rutherford 1980) is adequate to hydrolyze less than 1% of the cAMP present in our reaction mixture. In addition, the kinase reaction mixture contained dithiothreitol which inhibits PDE.

The cAMP-dependency observed in most of the tissue pieces should not be interpreted as meaning that the enzyme is inactive *in vivo* and activated only by the addition of exogenous cAMP in the reaction mixture. We have shown that the subunits of the holoenzyme will reassociate within a matter of minutes when cAMP is removed from extracts. Assuming a cAMP content of 10 fmoles in a tissue piece of 0.5 ug, (Merkle *et al.* 1984) addition of 29 ul of reaction mixture would result in a final cAMP concentration of 35 pM in the reaction volume. This concentration is too low to

sustain the dissociation of the subunits (Kd = 30 nM, Rutherford et al. 1982), which would then reassociate to form holoenzyme. Thus the lack of non-cAMP dependent activity should not be equated with in vivo inactivity. An alternative method would be required for determining the in vivo activation state of the kinase.

The result of finding cAMPdPK holoenzyme in both of the cell types throughout development is consistent with the idea that cAMP is involved with differentiation of both cell types. However, it is difficult to say to what extent the enzyme is active in vivo in either cell type. Even if the enzyme is present in stalk cells, there may be inadequate levels of cAMP to activate the enzyme. Therefore it is not possible to determine from these studies whether the cAMPdPK is performing a function that is cell-type specific or of a general nature. Presumably the enzyme is active in vivo at least in the prespore cells and tips of culminates since this is where cAMP levels are highest (Merkle et al. 1984).

Thus the control mechanism(s) for the cAMP response appear not to be determined by differential synthesis of the cAMPdPK, but in the differential activities of adenylate cyclase and phosphodiesterase and the resultant distribution of cAMP. The cAMPdPK therefore appears to act as a cAMP receptor, available in all cells but non-functional unless

activated by a cAMP message. However, there may exist numerous possibilities for localization of kinase function through differential distribution of protein substrates and phosphoprotein phosphatases. Many more experiments will be required to elucidate the total metabolic response of Disctyostelium cells to cAMP.

A similar argument can be made for a possible role of the cAMPdPK in pattern formation. Since it has been proposed that cAMP is a diffusable morphogen regulating pattern formation in slugs (Sternfeld and David 1981), it seems arguable that the receptor for cAMP (which may be the cAMPdPK), should be present in all cells of the pseudoplasmodia. Thus when slugs are bisected, altered levels of cAMP may be detected by the cAMPdPK which might then begin a series of metabolic processes resulting in the re-formation of correct cell proportioning.

Certainly the results of this study alone do not provide a complete explanation for cAMP action in Dictyostelium. However, as discussed by Wright (1966,1979), it is not possible to focus on a single event in a series and point to it as the sole cause of development. Rather, development involves a complex set of interactions encompassing changes in enzyme activities, levels of metabolites, substrates and inhibitors, as well as

alterations in the control of gene expression. Therefore the most important contribution of this study may be that it provides a piece of information to be used in conjunction with other information on metabolites, substrates, phosphatases, etc. In such a way it may ultimately be possible to elucidate the chain of events that exists in Dictyostelium between the primary signal of cAMP and the complex phenomena of cellular differentiation.

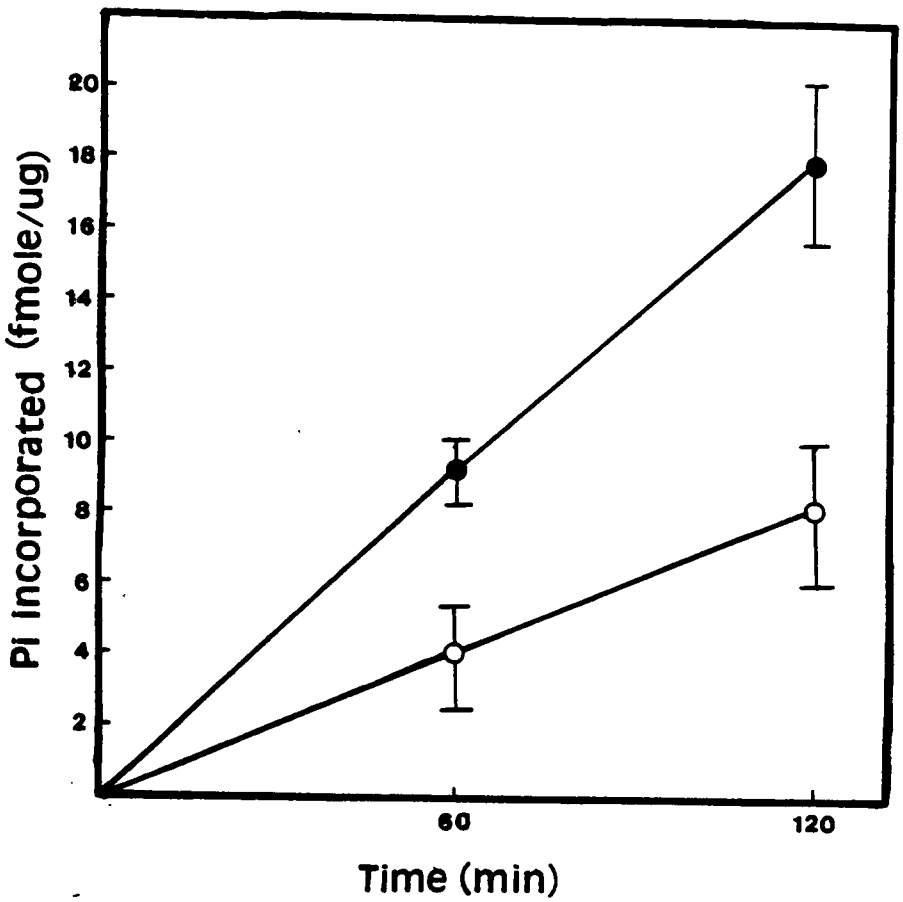


Figure 15. Linearity of cAMPdPK activity with time. Assays were performed using slug-stage tissue a described in methods in the presence (●) or absence (O) of cAMP. Each point is the average of triplicate determinations. The error bars indicate the standard deviation of each value.

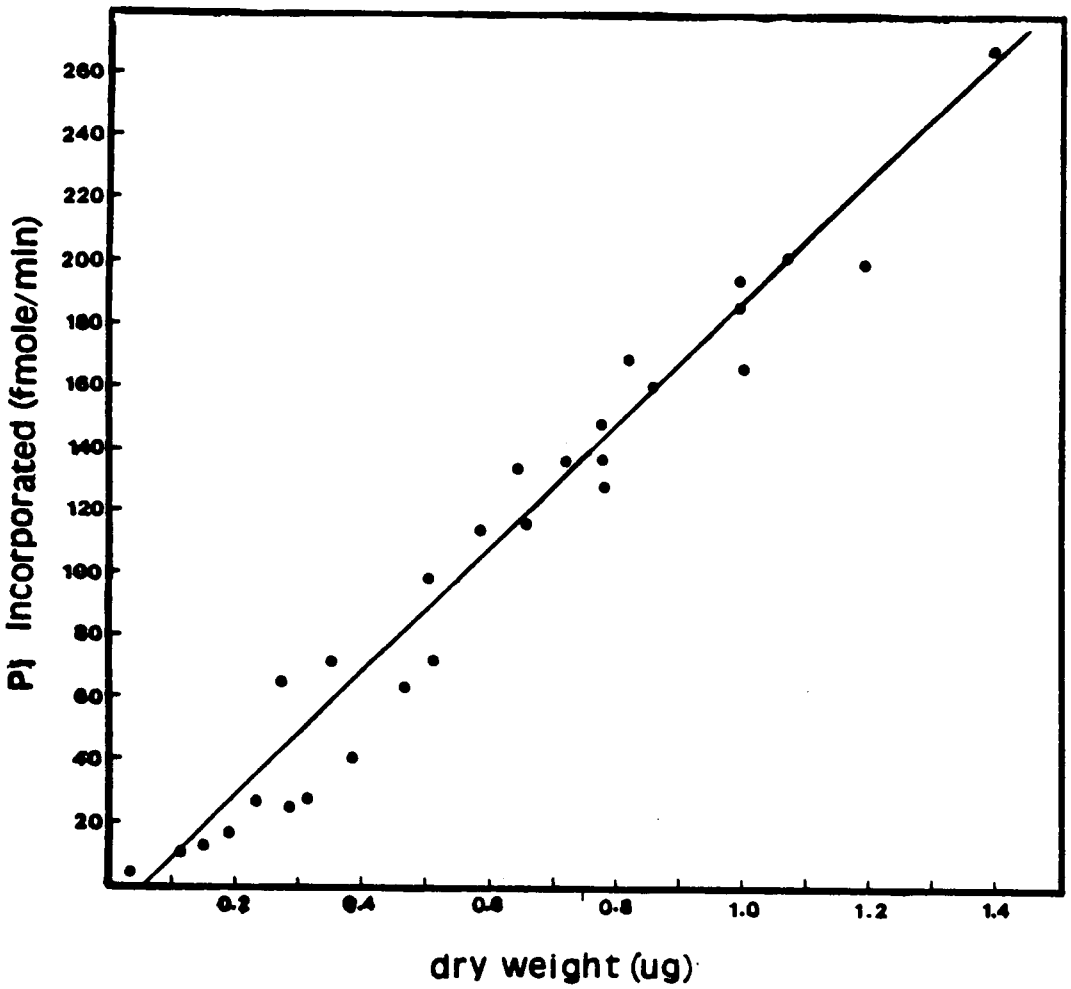


Figure 16. Linearity of Kemptide kinase reaction with tissue weight. Each point indicates the kinase activity of culmination stage tissue assayed in the presence of cAMP. The line was determined by linear regression of the data.

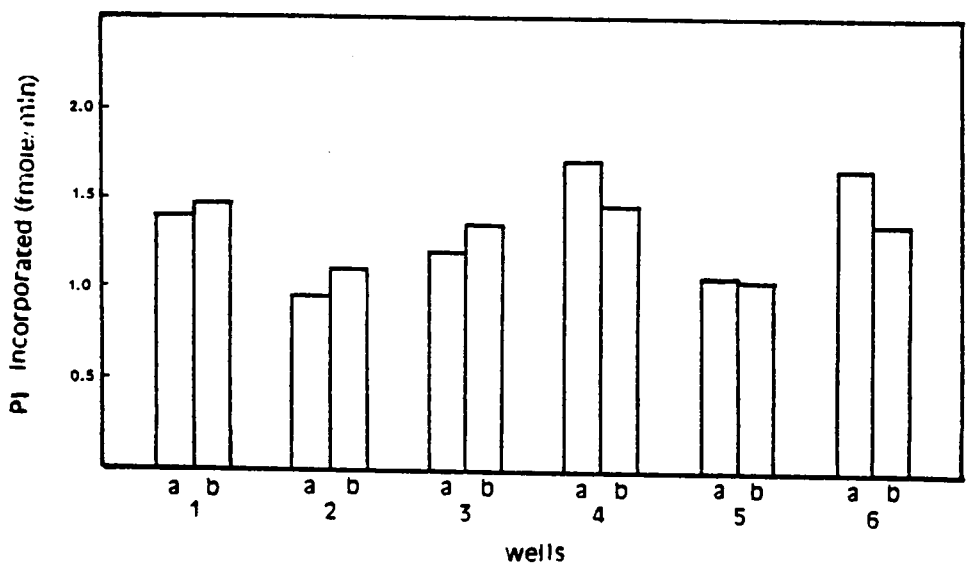


Figure 17. Solubility of Kemptide kinase. Sections of pseudoplasmodia were placed in the bottoms of well "a" of each pair of wells. Ten minutes after the addition of ereaction mixture half the volume was transferred to well "b" of each pair. The reactions were continued for the remainder of two hours and then processed separately as described.

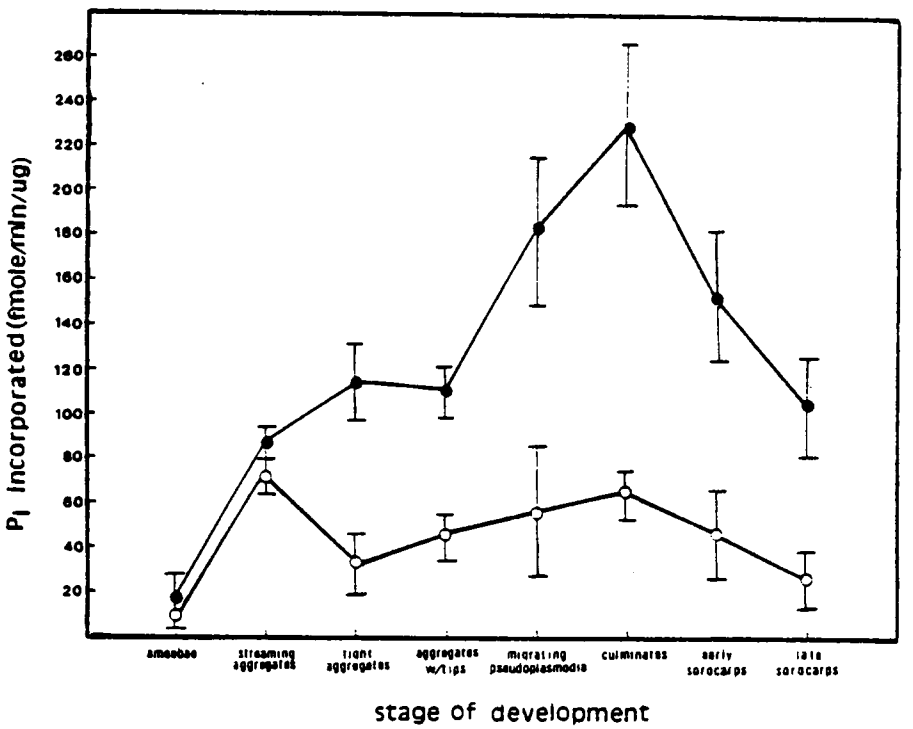


Figure 18. Kemptide kinase activity throughout development. Whole individuals at each indicated stage were bisected and one half was assayed for Kemptide kinase in the presence (●) of cAMP, the other half was asayed in the absence (O) of cAMP. For the assay of amoebae, starvation phase cells were plated on filters and lyophilized as described for the other stages. Freeze-dried sections of amoebae could then be weighed and assayed in the same manner. The error bars indicate the standard deviation of each determination. The number of individuals assayed at each stage was: amoebae, streaming aggregates, early and late sorocarps, n=4; aggregates after completion of streaming before (n=6) and after (n=6) tip formation; culminating individuals n=8; pseudoplasmodia, n=10.

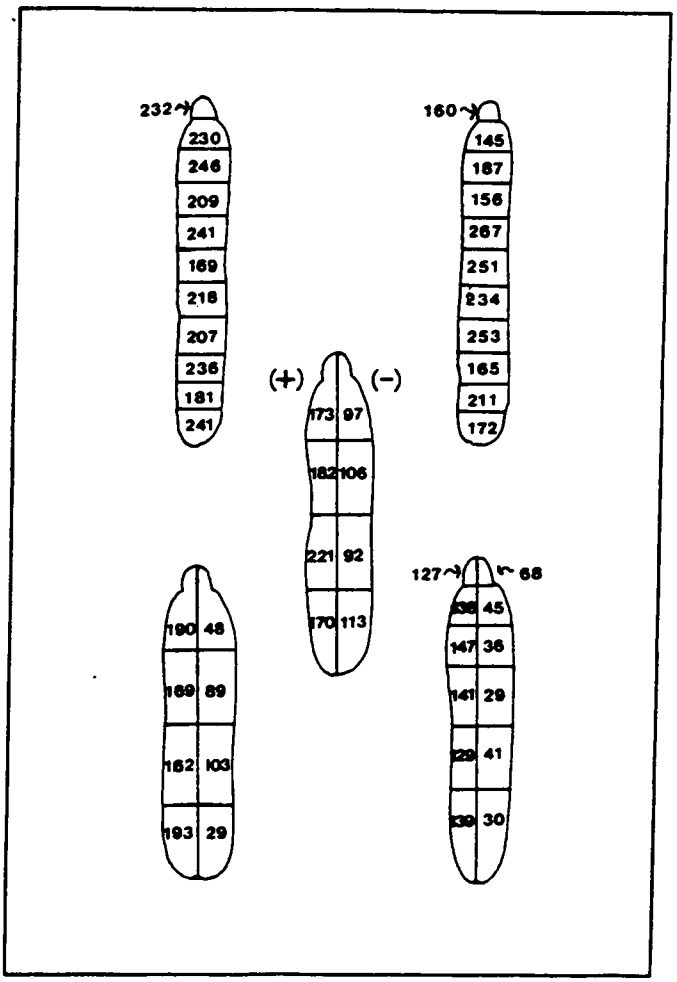


Figure 19. Distribution of Kemptide kinase in pseudoplasmodia. The two pseudoplasmodia represented on the top were assayed entirely in the presence of cAMP, the other three were assayed in the presence (sections on the left, indicated by +), or absence (sections on the right, indicated by -) of cAMP. The numbers represent units of kinase activity defined as fmoles phosphate incorporated per minute per µg dry weight. The pseudoplasmodia shown here are representative of a total of nine such determinations.

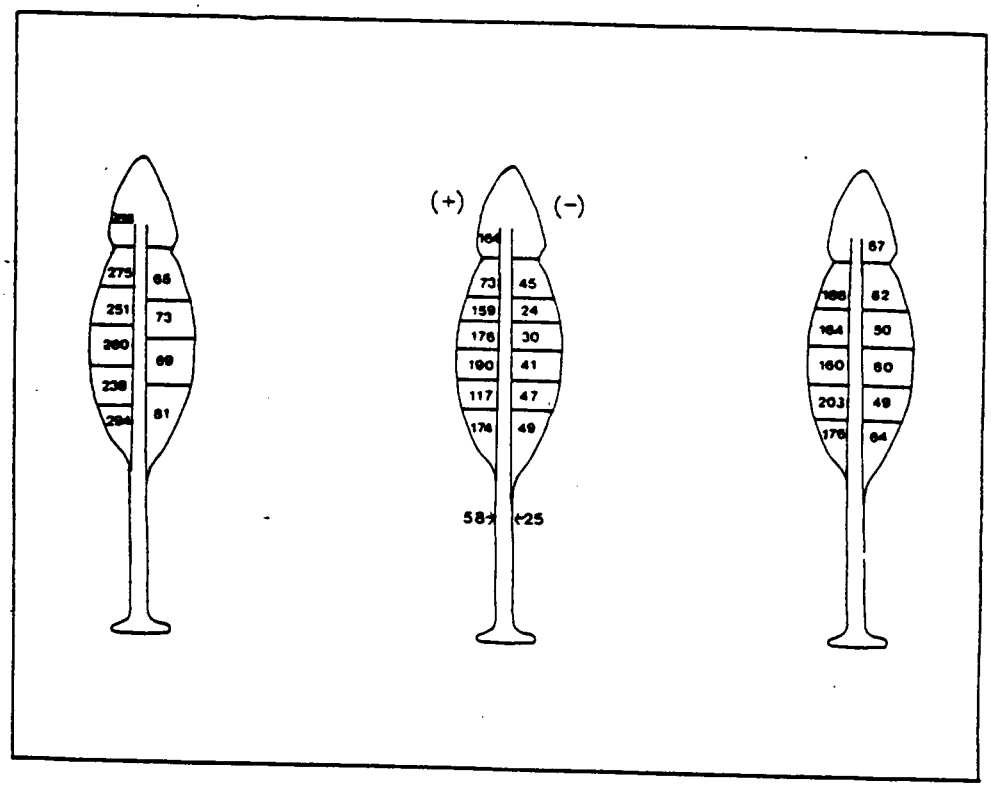


Figure 20. Distribution of Kemptide kinase in culminating individuals. Each individual was bisected through the midline, the stalk was removed, and the resulting prespore sections were dissected as shown. The sections shown on the left side of each individual (indicated by +), were assayed in the presence of cAMP, the sections on the right (indicated by -), were assayed in the absence of cAMP. The tips and stalks were assayed whole, either in the presence or absence of cAMP as described in the text. The numbers represent units of kinase activity (fmole/min/µg).

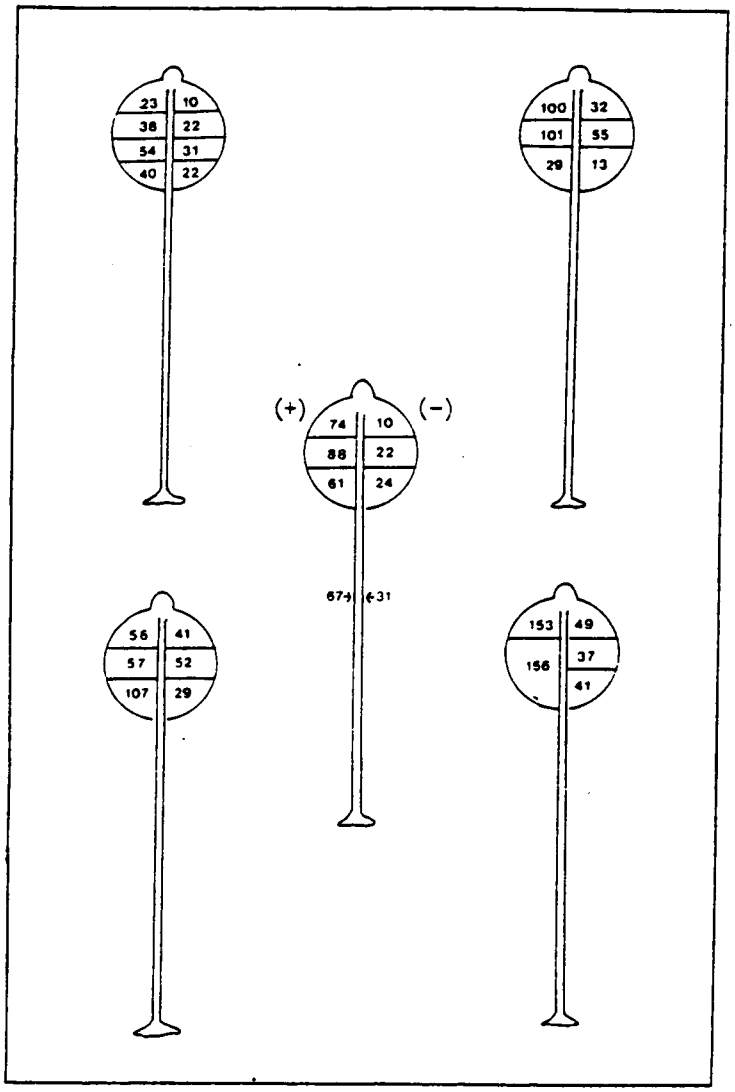


Figure 21. Distribution of Kemptide kinase in early sorocarps. Each individual was dissected as shown and assayed for Kemptide kinase in the presence (left,+) or absence (right,-) of cAMP. Stalks were assayed as described in the text.

Chapter 5 SUMMARY

The results presented in this work show that the cAMPdPK from Dictyostelium discoideum has many characteristics similar to the mammalian cAMPdPKs. The Dictyostelium enzyme is remarkably similar to the mammalian cAMPdPKs in terms of substrate specificity, Kms for ATP and protein substrates, affinity for cAMP, catalytic subunit Mr, pH optimum, and ion requirements. There appear to be differences in the Mr of the regulatory subunit, behavior on DEAE resin, and possibly in quaternary structure. The ease with which the subunits dissociate during ion-exchange chromatography and density gradient centrifugation indicates a certain amount of amino acid composition difference compared to the mammalian enzymes which retain the holoenzyme form during these treatments. A significant difference between the Dictyostelium and mammalian cAMPdPK is the apparent lack of the heat-stable protein kinase inhibitor that is found in mammalian cells (Walsh et al. 1971). The Dictyostelium enzyme can be inhibited by both the bovine heart and rabbit skeletal muscle inhibitor (not shown) but there does not appear to be a similar endogenous inhibitor in Dictyostelium.

The Dictyostelium cAMPdPK was further characterized with respect to properties used to distinguish the mammalian

type I and type II isoenzymes. This was done not only in the hopes that such a characterization might support a generalized function for the Dictyostelium kinase (i.e. proliferation or differentiation) but also because many of these properties are related to mechanisms that might modulate the activity of the kinase in vivo. Although the comparison of the Dictyostelium cAMPdPK to the mammalian isoenzyme did not provide a completely definitive answer to the relationship of the Dictyostelium enzyme and the mammalian prototypes, it was found that the Dictyostelium was similar to the type II isoenzyme in several properties. Since it is not completely clear whether the Dictyostelium cAMPdPK is more like type I or type II, perhaps the firmest statement about the Dictyostelium cAMPdPK which might proceed from this portion of the work would be the information on the possible modulatory mechanisms. Most interesting in this respect was the finding that histones are relatively effective substrates and can activate the kinase even in the absence of cAMP. It is certainly tempting to speculate that this cAMPdPK phosphorylates endogenous histones in vivo and functions in expression of late-stage genes. However, as yet there is no evidence in support of this idea, and determining whether the histone effect is significant in vivo will require further investigation.

The effects of pH and NaCl on the cAMPdPK are also difficult to assess without further investigation. It has been found that pH affects cell-type differentiation with stalk differentiation being promoted at pH 7.5 and spore differentiation favored at pH 6.2 (Gross *et al*. 1983). Between these pH values both the overall kinase activity and the ability of the subunits to reassociate are affected greatly, but whether or not the kinase is related to the pH effects *in vivo* is unknown. There is also a slight effect of physiological ion concentrations (150 mM), on the kinase and reconstitution, but the probability of this being physiologically significant seems small.

The mechanism by which cAMP is related to cellular differentiation in *Dictyostelium* is not known. Because it can induce both spore and stalk differentiation *in vitro*, cAMP alone does not appear to be sufficient to direct amoebae into a particular pathway of development. It may be that cAMP or cAMP induced processes undergo complex interactions with other morphogens to produce appropriate development. Other low molecular weight morphogens have been described in *Dictyostelium* and are currrently the objects of intensive investigation.

Ammonia (NH_3) has been proposed to be a morphogen in *Dictyostelium* based on its ability to suppress formation of

stalk cells and enhance formation of spore cells (Gross et al 1981,1983, Weeks 1983), while another substance, DIF (for stalk differentiation inducing factor), appears to promote stalk cell production (Gross et al. 1981, 1983, Town and Stanford 1979). Another low MW factor about which very little is known has recently been discovered and appears to be involved in the spore pathway (Weeks 1984).

Various hypotheses have been forwarded concerning the interactions of these factors with cAMP and how they might be responsible for the correct development and pattern in Dictyostelium. Studies by Weeks and colleagues have delineated a temporal sequence for the requirement of these substances for stalk cell differentiation (Neave et al. 1983, Sobolewski et al. 1983). In their system (V12M2 cells grown in monolayer) the ability of NH_3 to inhibit stalk cell differentiation occured very early (about 4 h after starvation), while the cAMP requirement for cell differentiation occured at about 8 h (coincident with tip formation in aggregates). It is interesting that cAMPdPK activity becomes detectable at approximately the same time. The DIF requirement occurred 1-2 h after the cAMP requirement and was not expressed in cells which had not been previously treated with cAMP. Therefore it may be that NH_3 and/or cAMP initiate developmental processes and the

cell-specific pathway is regulated by other morphogens. Certainly a great deal more work will be required to elucidate the various roles of each of these substances and their possible interactions with each other.

The results of finding cAMPdPK holoenzyme in both of the cell types throughout development is consistent with the idea that cAMP is involved with differentiation of both cell types. However it is difficult to say to what extent the enzyme is active in vivo in either cell type. Even though the enzyme is present in the stalk cells, there may be inadequate levels of cAMP to activate it. Presumably the enzyme is active in vivo at least in the prespore cells and tips of culminates since this is where cAMP levels are highest (Merkle et al.1984).

Several models have been proposed which implicate a gradient of cAMP or other morphogens in cell differentiation and/or pattern formation in Dictyostelium. Wolpert (1971) suggests that cells are assigned information by which they can interpret their position in a field and that cells differentiate in response to this information. Positional information is given by a linear gradient set up by "source" cells producing a diffusable substance and a "sink" at the other end of the gradient which destroys the morphogen (Crick 1970). The fact that a striking gradient of cAMP

occurs in culminating individuals is consistent with this mechanism. It may be that even though there is little localization of the cAMPdPK, there is differential activation of this enzyme in vivo in response to the cAMP gradient. In addition there may be differential distribution of substrates or phosphatases which may act to localize the activity of the kinase.

The full explanation of how and if the cAMPdPK works within the cell to mediate developmental events will certainly require the identification of the enzyme's endogenous substrates. Many studies have investigated both in vivo and in vitro phosphorylation in Dictyostelium. Weinstein and Koritz (1973) demonstrated a membrane bound non-cAMP dependent protein kinase which had equal activity in vegetative and developing cells, while another non cAMP dependent protein kinase was found in plasma membranes of vegetative cells but decreased in activity upon starvation. (Juliani and Klein 1981). A ribosomal protein was found to undergo phosphorylation during the aggregation stage (Juliani et al. 1983). Cyclic AMP stimulation of cells results in the increased activity of a myosin light chain kinase which is believed to regulate contractile elements involved in the chemotactic response (Malchow et al. 1981). Coffman et al. (1981,1982) reported phosphorylation of

cytosolic and membrane proteins, some of which were synthesized prematurely in response to cAMP. Two of the phosphoproteins were later determined to be spore coat proteins (Delaney et al. (1983). Lubs-Haukeness and Klein (1982) reported cAMP-dependent phosphorylation of a 47,000 dalton membrane protein. Frame and Rutherford (1984) investigated the in vitro cAMP-dependent phosphorylation of cytosolic proteins and identified several potential endogenous substrates for the cAMP dependent kinase. However, to date there has been no identification of a cellular process regulated by cAMP-dependent phosphorylation.

Another aspect of the overall picture of cAMP metabolism may be the presence of phosphatases which would counteract the phosphorylation of endogenous substrates. Several phosphatases, including Kemptide and histone phosphatases, have been shown to exist in Dictyostelium (Ferris, Naranan, and Rutherford, manuscript in preparation) and may provide such a mechanism for counter-regulation.

By analogy to mammalian systems several possible functions for the cAMPdPK can be suggested. One possibility may be that the kinase regulates intracellular glycogen metabolism. This is an interesting idea because of the role of glycogen metabolism in Dictyostelium. During culmination

glycogen degradation in Dictyostelium occurs as a means of providing glucose units for incorporation into the cellulose used in the stalk sheath (Raper and Fennell 1952). The work of Rutherford and Harris (1976) showed maximum glycogen phosphorylase activity in the tip region of culminates and decreasing activity in the cells further down the stalk. High levels of cAMP in the tip region activating the cAMPdPK might then either directly or indirectly activate glycogen phosphorylase via phosphorylation. Such a hypothesis is compatible both with the results from this laboratory and the analogous situation in mammals where glycogen is degraded in response to the second messenger cAMP. Only recently in this laboratory have two forms of glycogen phosphorylase been discovered in Dictyostelium. Investigation of a link between these two forms and the cAMPdPK is underway. Other studies in this laboratory are underway to investigate a possible connection between the cAMPdPK and the two forms of glycogen synthetase.

Another possible role for the cAMPdPK may be in regulating chemotaxis of cells during the morphogenetic movements of culmination. Selective phosphorylation of cytoskeletal elements and/or membrane proteins might result in the responses of prestalk/prespore sorting or differential adhesion which are hypothesized to produce correct patterning.

Finally, the cAMPdPK may play a part in the differential transcription or translation of mRNAs leading to the appearance of late-stage and/or cell-type specific proteins and enzymes. This control could conceivably occur at one or more levels - phosphorylation of histone or non-histone chromosomal proteins resulting in altered gene activity, phosphorylation of RNA polymerase resulting in differential transcription of genes, or phosphorylation of ribosomal proteins resulting differential translation of mRNAs. This particular function would be consistent with the studies showing differential synthesis and stability of late-stage mRNAs.

Determining which of these functions, if any, are performed by the Dictyostelium cAMPdPK may help shed light on the mechanisms of cell differentiation or pattern formation. Such information in turn, may be relevant in the study of other types of organisms. Since a common theme in nature is the conservation of effective processes, elucidation of a developmental mechanism in Dictyostelium may also have applicability for other areas of developmental biology.

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