A Study of the Regulation of Glycogen Metabolism

in Dictyostelium discoideum

by

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(ABSTRACT)

This work discusses the regulation of glycogen metabolism in *Dictyostelium discoideum* during its developmental cycle. Specifically, the possible cAMP dependent regulation of glycogen phosphorylase and glycogen synthase was examined. In other systems, cAMP can regulate at the level of the gene (procaryotes, CAP protein) or at the level of covalent, reversible modification of the enzyme activity (eucaryotes, cAMP-dependent protein kinase-cAMPdPK). In *Dictyostelium*, glycogen phosphorylase and glycogen synthase have each been found to occur in two forms; one regulated allosterically and the other independent of allosteric regulation.

The regulation of the two forms of glycogen phosphorylase was examined in single-cell suspensions to which cAMP or one of several cAMP analogs were added to mimic differentiative conditions. The allosterically regulated form of glycogen phosphorylase, phosphorylase *b*, decreased in the presence of cAMP while a corresponding increase in phosphorylase *a*, the non-allosterically regulated form of glycogen phosphorylase, was observed over an 8 hr period in the same cultures. In the presence of cAMP analogs a similar time course of regulation for the two forms of glycogen phosphorylase occurred but only 2′deoxy-cAMP gave an effect comparable to cAMP. Under these same conditions, northern blot analysis of three developmentally regulated mRNAs--PL3, D11, and D3--revealed that normal gene regulation was occurring. Under conditions where elevation of intracellular cAMP was inhibited, neither regulation of phosphorylase enzyme activity nor of the 3 genes was observed. This indicated that under these conditions intracellular elevation of cAMP was necessary for the observed effects on enzyme and gene activity. This requirement for intracellular cAMP may indicate the involvement of a cAMPdPK.

The properties of a phosphorylase *b* kinase found in amoebal extracts are described. The kinase activity coeluted with the phosphorylase *b* activity on a DE-52 anion exchange column. Under the conditions described conversion of the phosphorylase *b* activity to the phosphorylase *a* activity was observed. However, an increase in molecular weight to 104 kd (as seen for purified phosphorylase *a*) was not observed.

The characterization of a partially purified glycogen synthase *I* and its developmental regulation are described. Also described are *in vitro* attempts to convert the *I* form to the allosterically regulated, *D* form, under conditions conducive to phosphorylation.

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I would first like to express my love and appreciation for my family. They have supported me through the good times and the bad times of my graduate work. I would like to thank Dr. Rutherford for giving me the opportunity to work in his lab. Dr. Rutherford's patience and guidance helped me to become a better research scientist. He set an excellent example of what a good researcher should be--persistent and always seeking. Special thanks goes to my committee members for their patience and support even when data was lean. To my lab mates of my dissertation years-- , , , , , , , , and --thank you for your help, your advice, and most of all for your laughter.

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1.0 Introduction

A multicellular organism is composed of a few to many cell types cooperating toward the survival of the organism. This is a simple concept, yet each of these cell types arose from a single, undifferentiated cell; by a decidedly complex process. The differentiation and morphogenesis required to produce a multicellular organism has been studied for decades. However, it was not until recently that the biochemical and molecular biological techniques necessary for a thorough investigation of differentiation became available. A further requirement for such studies is a relatively simple model system, one that eliminats as many extraneous processes as possible from that of differentiation. *Dictyostelium discoideum* has proven to be one of the best models fitting this requirement. *Dictyostelium's* developmental cycle does not involve growth or cell division, thereby, eliminating two processes commonly interfering with the investigation of cellular differentiation. Further, *Dictyostelium* provides the simplicity of only two spatially-separated cell types, as well as an easily grown and synchronized cell population.

1.1 The Life Cycle of Dictyostelium discoideum

Dictyostelium discoideum is a cellular slime mold commonly found in the leaf litter of temperate forests the world over (Raper 1935). The amoebae grow and multiply while feeding on bacteria in the leaf litter until the area is depleted. As starvation begins, the developmental cycle is initiated as a survival mechanism for a portion of the population (Figure 1; reviewed in Loomis, 1982).

The starvation stimulus causes the amoebae to synthesize a chemotactic system sensitive to cAMP including cAMP receptors, adenylate cyclase--for cAMP synthesis, and cAMP phosphodiesterase--for cAMP degradation, as well as the required regulatory molecules. Scattered throughout the population, certain amoebae begin to emit pulses of cAMP. Surrounding amoebae begin moving toward this "aggregation" center while relaying the cAMP signal outward in a wavelike pattern of synthesis and degradation (Konijn et al. 1968). After 7-10 hr, the cells have formed a mound of $\simeq$ 100,000 cells. Cell-cell contacts are made and essentially form temporary multicellular organism or "cooperation". The cells of this "cooperation" continue to mound up until the slender "finger" stage develops. The "finger" stage falls over to begin the migratory phase of the life cycle as the slug or pseudoplasmodium. For the first time in the developmental cycle, two cell types can be distinguished. The two cell types were first distinguished and their developmental paths traced using vital dyes (Bonner 1952; Durston and Vork 1979; MacWilliams and Bonner 1979). The anterior one-third of the slug stained very darkly with the dyes. Tracing the developmental path of these darkly staining cells showed them to eventually form stalk cells. For this reason, the red staining cells of the slug are called prestalk cells. The non-staining cells composing the posterior two-thirds of the slug were destined to form the spore cells and were therefore called prespore cells. Environmental factors, most importantly light and moisture, determine the length of slug migration. Normally, the slug ceases to migrate at 18-20 hours into the life cycle. The slug sits back upon itself with its tip of prestalk cells uppermost. The prestalk cells begin to migrate down through the prespore cell mass in what has been described as a "reverse fountain motion". The prestalk cells begin depositing

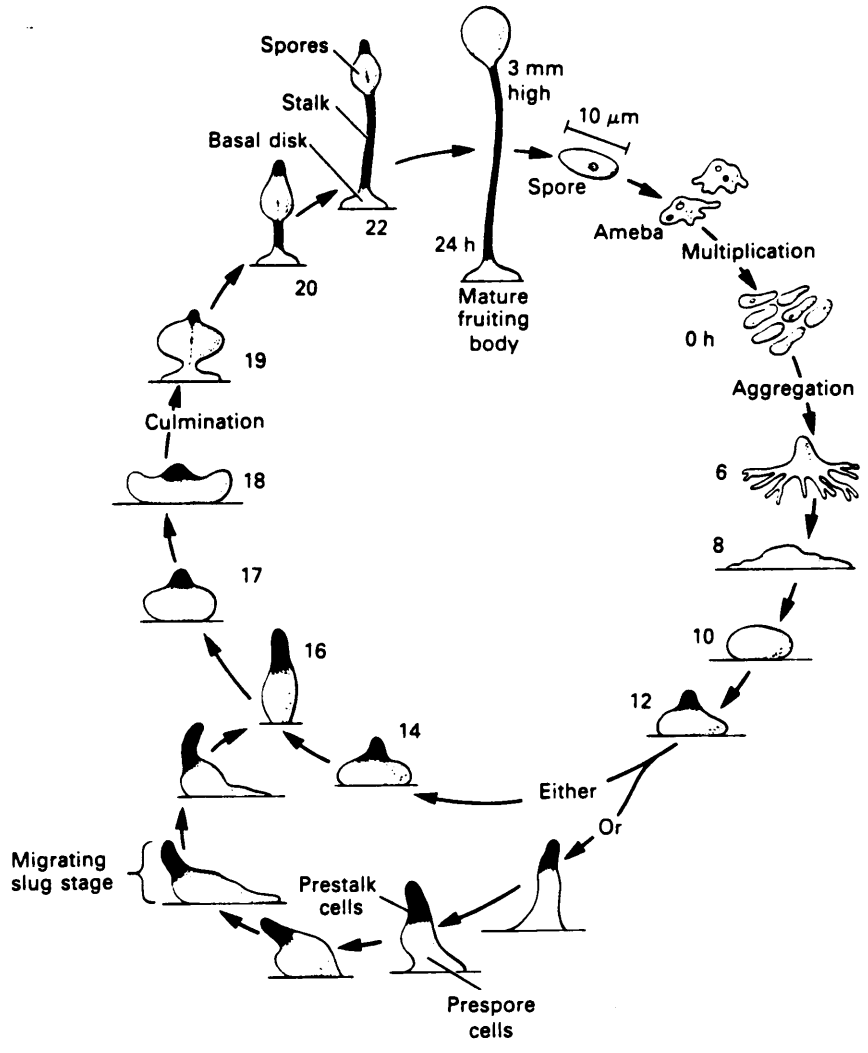

Figure 1. The Life Cycle of Dictyostelium discoideum

a cellulosic sheath as they migrate downward literally lifting the spore mass off the substratum. The culmination stage completes the life cycle as the stalk cells become vacuolated and their cell walls thicken to support the prespore mass. The prespore mass then terminally differentiates into spores completing the life cycle $\simeq$ 24 hr after aggregation began. Each spore of the spore mass can release, under favorable conditions, a single amoeba capable of repeating the entire life cycle.

1.2 The Role of cAMP During Differentiation of the Two Cell Types

In addition to functioning as a chemotactic agent during development, cAMP is also involved in the differentiation of both prestalk and prespore cells. Several studies have shown cAMP levels to be elevated intracellularly during development (Malkinson and Ashworth 1973; Pahlic and Rutherford 1979; Merkle et al. 1984). However, there is some controversy as to the relative distribution of cAMP between the two cell types during development. The studies mentioned above did not find any differential distribution of cAMP between the two cell types. However, Brenner (1977) found that in migrating slugs, prestalk cells contained from 40%-70% higher concentrations of cAMP than the prespore cells. Cyclic AMP had been shown to stimulate the differentiation of stalk cells when applied to wild-type amoebae (Bonner 1970). Feit et al. (1978) demonstrated the involvement of cAMP in the differentiation of both cell types by implanting cAMP soaked Sephadex beads in slugs. Both stalk and spore cells differentiated in the area around the implanted beads. This evidence supported the studies with stalky and sporogenous mutants which differentiated into stalk or spore cells upon exposure to high levels of cAMP (Town et al. 1976; Kay et al. 1978). However, cAMP is not the only factor involved in differentiation; a "differentiation inducing factor" (DIF) is also required for stalk cell differentiation (Town et al. 1976; Kay and Jermyn 1983). Other factors such as ammonia may also be involved (reviewed in Loomis 1982). Thus, cAMP seems essential to the differentiation of both cell types but it is not specific nor the only requirement for the differentiation of either cell type.

Cyclic AMP levels may also be involved in morphogenesis and in the maintenance of developmentally regulated mRNAs. Several studies have proposed that the chemotactic responsiveness of the cells may account for the morphogenetic movements which occur during development (Maeda and Maeda 1974; Sternfield and David 1981; Matsukuma and Durston 1979). Thus, the gradient of cAMP concentration in the slug observed by Brenner (1977) may not only function in differentiation of cell types but also in morphogenetic movements. Recently many cAMP regulated genes have been isolated and their regulation during development and in response to exogenously added cAMP and cAMP analogs have been investigated (reviewed in Spudich 1987). The work leading to the isolation of developmentally regulated genes involved the observation that certain mRNAs could be reinduced in disaggregated slug cells by the addition of cAMP but remained degraded in the absence of cAMP (Barklis and Lodish 1983; Blumberg et al. 1982; Mehdy et al. 1983). Other workers had previously found that cAMP was required for the synthesis and maintenance of many developmentally regulated enzymes and late stage mRNA's (Gross et al. 1981; Kay et al. 1979; Landfear and Lodish 1980; Town and Gross 1978).

1.3 Possible Modes of Action For cAMP

Cyclic AMP has been recognized as a regulatory molecule in both eucaryotes and procaryotes. In procaryotes, it regulates gene activity in conjunction with a cAMP binding protein (CAP protein) through direct interaction with the DNA (Darnell et al. 1987). In eucaryotes, it seems to act as a second messenger for the activation of a cAMP-dependent protein kinase (cAMPdPK). Experimental results in *Dictyostelium* could be interpreted to support either hypothesis. In 1982, the identification and characterization of a cAMPdPK in *Dictyostelium* (Rutherford et al. 1982; Leichtling et al. 1982; de Gunzburg and Veron 1982; Cooper et al. 1983; Schoen et al. 1983) suggested that cAMP acts as a second messenger as in other eucaryotic systems. The many intracellular effects would then be mediated through phosphorylation of intracellular substrates. However, an intracellular substrate has not yet been identified. Recent work using

cAMP analogs and inhibitors of adenylate cyclase has given further support to the procaryotic-like mechanism proposal. These recent studies (Oyama and Blumberg 1986a,b,c; Kimmel 1987; Haribabu and Dottin 1986) have given evidence toward the possible interaction of cAMP with the membrane receptor as the link between extracellular cAMP and its intracellular effects. Lending further support to this hypothesis was the recent finding by Kay et al. (1987) for the translocation of the cAMP receptor into the nucleus. This may, at the least, explain cAMP effects on gene actvity.

1.4 The Regulation of Glycogen Metabolism in Dictyostelium

Glucose and its polymeric storage form, glycogen, are used in both procaryotes and eucaryotes as a source of energy and as a source of precursor molecules for the synthesis of various polymeric structures. During the development and differentiation of *Dictyostelium*, glycogen is not used as an energy source (breakdown of proteins provides the required energy) but as a source of precursor molecules for the synthesis of the prestalk cells' cellulosic cell walls, mucopolysaccharide for the thick spore coats, and trehalose.

The two enzymes involved in the synthesis and degradation of glycogen--glycogen synthase and glycogen phosphorylase--are each found in two easily distinguishable forms in *Dictyostelium* (Rosness et al. 1971; Rutherford and Cloutier 1987). Glycogen levels are low in the growing amoebae but increase to high levels upon induction of differentiation by starvation and the resulting increase in cAMP. The essential nature of both cAMP and glycogen to the differentiation of both cell types leads to the hypothesis that the regulation of glycogen metabolism is through cAMP. This possible regulation may be at the level of enzyme activity as occurs in other eucaryotes or at the level of gene activity.

2.0 The Regulation of the Two Forms of Glycogen Phosphorylase by cAMP and Its Analogs in Dictyostelium discoideum

Abstract

We have recently reported the existence of two forms of glycogen phosphorylase in *Dictyostelium discoideum*. During normal development the activity of the glycogen phosphorylase *b* form decreased as the activity of the phosphorylase *a* form increased. The total phosphorylase activity remained constant. The physical and kinetic properties of the *Dictyostelium* enzyme were similar to those of the mammalian enzyme. In mammals, cAMP regulates the conversion of the two forms by a cAMP dependent protein kinase (cAMPdPK). We report here that if cAMP is added to a single cell suspension, the *Dictyostelium* phosphorylase is converted from a 5'AMP dependent *b* form to an independent *a* form. We also show the effect of several cAMP analogs on the phosphorylase activity in these single cell suspensions. The cAMP analogs were selected on the

basis of their affinities for the membrane-bound cAMP receptor or the cytoplasmic cAMPdPK. We found that relatively low levels, 100 μM , of cAMP or 2'd-cAMP added to aggregation-competent cells in shaking culture caused a loss of phosphorylase *b* activity and the appearance of phosphorylase *a* activity. The analog, 2'd-cAMP, has a high affinity for the cAMP receptor but a low affinity for the cAMPdPK. Two other analogs, Bt_2-cAMP and 8-Br-cAMP, which have low affinities for the cAMP receptor but high affinities for the cAMPdPK, required high levels (500 μM) for *b* to *a* conversion. cDNAs to three cAMP-regulated genes--PL3,D11, and D3-- were used as controls in the above experiments. In order to determine if intracellular levels of cAMP were involved in the regulation of phosphorylase activity, both the phosphorylase and the PL3, D11, and D3 mRNA levels were examined in cells suspended in a glucose/albumin mixture--a medium in which adenylate cyclase is inhibited. Under these conditions, neither gene regulation nor a change in the phosphorylase *b* to *a* activity occurred in response to added extracellular cAMP. The results suggest that an intracellular increase in cAMP is involved in the regulation of the two forms of glycogen phosphorylase in *Dictyostelium.*

2.1 Introduction

Cell differentiation in *Dictyostelium* follows a dual pathway resulting in the differentiation of two cell types. This developmental cycle provides an excellent system for studying differentiation without the added complexity of growing and dividing cells. The developmental cycle is initiated by removal of nutrients at which point cell growth and division ceases as differentiation proceeds (reviewed in Loomis, 1975). The two differentiated cell types, stalk cells and spore cells, have thick cell walls composed primarily of cellulose. Synthesis of this cellulose uses the glucose derived from the degradation of cellular glycogen by glycogen phosphorylase (Wright & Dahlberg 1967; Wright et al. 1968). This metabolic pathway has been studied extensively due to its relative simplicity and its direct involvement in the differentiation of both cell types (Konijn et al. 1968; Bonner et al. 1968; Rutherford et al. 1985).

In most eucaryotic systems, glycogen phosphorylase occurs as two forms interconvertible through a phosphorylation-dephosphorylation cycle. The non-phosphorylated *b* form is dependent on the presence of 5'AMP for activity, while the phosphorylated *a* form is not dependent on 5'AMP. The two forms are regulated by cAMP as mediated by a cAMPdPK (Fisher & Krebs 1955; Cohen 1978). Previous investigations indicated that the *Dictyostelium* enzyme was not regulated by cAMP because only one form of the phosphorylase was observed (Jones & Wright 1970; Firtel & Bonner 1972; Rutherford & Harris 1976; Higgins & Dahmus 1982; Thomas & Wright 1976a & b) and the existance of a cAMPdPK was in doubt (Rahmsdorf & Gerish 1978; Veron & Patte 1978). It was therefore concluded that glycogen phosphorylase in *Dictyostelium* was similar to the plant glycogen phosphorylases which only occur in one form (Thomas and Wright 1976a & b). More recently, Takeuchi and his coworkers (Takeuchi & Sakai 1971; Takemoto et al. 1978; Okamoto & Takeuchi 1976) have shown that addition of cAMP to shaking cell cultures induced the appearance of a glycogen phosphorylase activity that was not dependent on 5'AMP for activity. In their investigations, however, glycogen phosphorylase *b* was not assayed.

The discovery of cAMPdPK in *Dictyostelium* (Rutherford et al. 1982; de Gunzburg & Veron 1982; Leichtling et al. 1982; Schoen et al. 1983), renewed our interest in investigating the regulation of glycogen phosphorylase by cAMP. We recently reported on the identification and characterization of a second form of glycogen phosphorylase (Rutherford and Cloutier 1986; Cloutier and Rutherford 1987). This form, unlike the previously described enzyme, was dependent upon 5'-AMP for activity. The activity of the dependent form was maximal in growing amoebae and decreased during subsequent development.

Because previous investigators had shown an effect of added extracellular cAMP on one form of phosphorylase activity in *Dictyostelium* (Takemoto et al. 1978; Okamoto & Takeuchi 1976), we were interested in the relationship of the two forms of the enzyme under these conditions. In addition, recent reports have shown evidence for the direct involvement of the cAMP receptor in the regulation of several cAMP inducible genes (Kimmel 1987; Oyama and Blumberg 1986a,b & c; Haribabu & Dottin 1986). In this report we investigate the effect of several cAMP analogs, cGMP, cIMP, phorbol 12-myristate 13-acetate (PMA), and glucose-albumin medium on the regulation of the two forms of the enzyme, as well as three previously described cAMP regulated genes.

2.2 Materials and Methods

2.2.1 Materials

Cyclic 3′,5′-adenosine monophosphate (cAMP), 8-bromo cAMP (8Br-cAMP), dibutyryl cAMP (Bt_2-cAMP), monobutyryl cAMP (Bt-cAMP), 2′deoxy cAMP (2′d-cAMP), cyclic 3′,5′-guanosine monophosphate (cGMP), cyclic 3′,5′-inosine monophosphate (cIMP), phorbol 12-myristate 13-acetate (PMA), and other chemicals were obtained at the highest purity available from Sigma Chemical Co., St. Louis, MO. Agarose was obtained from International Biotechnologies, Inc., New Haven, CN. Nitrocellulose was purchased from Schleicher and Schuell, Inc., Keene, NH. Ultrapure phenol was from Bethesda Research Laboratories, Bethesda, MD. ^{32}P-CTP and ^{32}P-ATP were obtained from Dupont (New England Nuclear), Wilmington, DE.

2.2.2 Methods

2.2.2.1 Growth and Cell Differentiation

Dictyostelium discoideum, axenic strain Ax-3, was grown in HL-5 medium as described previously (Sussman and Sussman 1967). Cells were grown to a density of 5-10 x 10^6 cells/ml (late log phase), harvested by centrifugation, and washed twice in 7mM N-morpholinoethanesulfonic acid (MES) pH 6.5, containing 5 mM $MgSO_4$ and 20 mM KCl (Buffer A). The cell pellet was diluted 1:3 (weight:volume) in Buffer A, then evenly spread onto nonnutrient agar and placed at 7°C for 16 hr to obtain aggregation competency. No aggregation or rippling was observed at the end of this period. The aggregation-competent cells were removed from the surface of the nonnutrient

agar with Buffer A, then washed twice in Buffer A containing 1 mM K_2HPO_4/NaH_2PO_4 (Buffer B). The cells were diluted to 5 x 10^6 cells/ml in Buffer B, then distributed to 150 ml Erlenmeyer flasks (30 ml/flask). The flasks were shaken at 160 rpm on a gyratory shaker (Junior Orbital Shaker, LAB-LINE Inst.) for 8-10 hr at 23°C either in the presence or absence of cAMP or other additions described in the figure legends. Samples of the culture (5-10 ml) were removed for glycogen phosphorylase assays. The samples were either sonicated for immediate assay or frozen at -70°C. Samples (20 ml) were removed for total RNA purification at the times indicated in the figures.

Cyclic AMP or its analogs were added to the shaking cells at a final concentration of 0.5-1 mM unless otherwise indicated in the figure legend. After shaking for 4 hr, the cells were centrifuged, and resuspended in Buffer B and placed back on the shaker for the remainder of the experiment. Cyclic AMP or the other components were added to their original concentrations.

Glucose/albumin medium (GA) consisted of 5% glucose/2% albumin in Buffer B. This medium had been previously shown to inhibit adenylate cyclase activity (Oyama and Blumberg 1986a).

PMA in dimethyl sulfoxide (DMSO) was added at a final concentration of 100 ng/ml. Cells treated with the same percentage of DMSO alone served as a control. No effect on the glycogen phosphorylase activity or gene activity was observed in response to DMSO at the concentration used. Cells treated with PMA were harvested and treated in the same manner as cells treated with cAMP.

2.2.2.2 Sample Preparation and Glycogen Phosphorylase Enzyme Assay

Cells were harvested from the shaking flasks by centrifugation, then washed once in Buffer C (50 mM Tris-HCl pH 7.5, 0.02% sodium azide, and 2 mM Benzamidine). The cells were resuspended in 2 ml of Buffer C and ruptured by freezing or by sonication with two, 30 sec treatments using a 0.5 cm probe of a cell disruptor (Virtis or Fisher Model 300). Frozen cells were thawed at

room temperature, then mixed with a pasteur pipette to promote cell breakage and centrifuged at 10,000 x g for 6 min.

A 20 μl sample from the 10,000 x g supernatant was added to 200 μl of a reaction mixture containing 50 mM imidazole (pH 6.8), 2.5 mg/ml glycogen, 5 mM $MgCl_2$, 0.5 μg/ml glucose-1,6-diphosphate, 2 mM K_2HPO_4, 0.3 units/ml glucose-6-phosphate dehydrogenase, and 0.4 units/ml phosphoglucomutase. The temperature of the assay mixture was maintained at 23°C. A molar extinction coefficient of 6.2 x 10^3 was used to quantitate NADPH formation at 340 nm. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol NADPH/min at 23°C.

2.2.2.3 SDS-PAGE and Western Blotting of Proteins

Protein samples were prepared for SDS-PAGE by boiling in the presence of 2% SDS, 5% DTT and 0.02% Pyronin Y. The samples were separated on 6% SDS-PAGE according to Laemmli and Favre (1973). Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes using a Hoefer transfer chamber and power source (Model TE51) set at 1.2 amps for one hour. The transfer buffer contained 192 mM glycine and 20% methanol in 25 mM Tris-HCl, pH 8.3. After transfer, the nitrocellulose was placed in 50 ml of 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, and 0.1% Tween 20 (Buffer D), and gently shaken for 20-30 min (Immunostaining method was a personal communication from Dr. Asim Esen, Biology Dept., Virginia Tech, Blacksburg, VA.). The buffer was then replaced with antiserum diluted 1/500-1/170 in fresh Buffer D. After 12 hr of incubation with the antibody, the nitrocellulose was rinsed several times with fresh 50 ml solutions of Buffer D. The blot was transferred into a 50 ml solution of Buffer D containing 1 μg/ml protein A-peroxidase and then incubated with gentle shaking for 1 hr. The nitrocellulose was rinsed with two 50 ml volumes of Buffer D and then with 50 ml of Buffer D lacking Tween 20 (Buffer D'). The nitrocellulose was then exposed to 48 ml of peroxidase re-

action mixture containing 17% methanol, 0.5 mg/ml of 4-chloro-1-naphthol, and 0.008% H_2O_2 in Buffer D' for 30-120 min.

2.2.2.4 Antibody Preparation

Both the *a* and the *b* forms of the phosphorylase were purified to homogeneity as described previously (Cloutier and Rutherford 1987). These proteins were subjected to preparative SDS-PAGE, then were eluted from the gel slices electrophoretically using an ISCO electroeluter (Model 1750 Sample Concentrator). Two New Zealand white rabbits were each injected with 50-150 μg purified protein in complete Freunds adjuvant. The rabbits were reinjected with 50-100 μg purified protein in incomplete Freunds adjuvant at two week intervals. After 6 weeks, the presence of antibody to the protein could be detected by western blotting. Whole blood was allowed to clot overnight at 4°C, then centrifuged at 10,000 x g for 10 min. The serum was divided into 300 μl aliquots and stored frozen at -20°C for subsequent use. Both antiserum preparations had similar titers; a 1:500 dilution of each antiserum was used for immunostaining and immunoprecipitation.

2.2.2.5 Recombinant Plasmid Probes

Three cDNAs--PL3, D11, and D3--inserted into plasmid pBr322 were gifts from Daphne Blumberg (National Cancer Institute, Frederick, MD). The PL3 insert is 2 kb and represents a prespore specific mRNA, the D11 insert is a prestalk enriched mRNA of 1 kb and the D3 is a growth specific mRNA of 0.9 kb (Oyama & Blumberg 1986a, b & c). The plasmids were purified by a modified SDS-NaOH lysis procedure according to the method of M. Tigges, University of California, Berkeley, CA (personal communication).

These plasmids were labelled according to the method of Maniatis (1982), except that we used the Amersham nick-translation enzyme, which is a mixture of DNA polymerase I and DNase I. Approximately 2 x 10^7 cpm of the nick-translated plasmids were used for the hybridization to the RNA transferred to each filter. The entire plasmid was used for nick translation and hybridization. We found that a labelled, uninserted pBr322 plasmid sample was unable to hybridize with total mRNA from *Dictyostelium* and therefore, hybridization was due to binding of the insert alone.

2.2.2.6 RNA Isolation and Analysis

Total RNA was isolated according to the method of Mehdy et al. (1983). Cells were pelleted, washed once in cold 50 mM Tris-HCl pH 8.4, and suspended to a concentration of 1 x 10^8 cells/ml. An equal volume of 50 mM Tris-HCl pH 8.4 containing 2% SDS was prepared in a separate tube. In a third tube, 2x the original cell volume of phenol/chloroform was prepared. Diethyl pyrocarbonate (DEPC) was added at a 1% concentration to the cells and to the Tris-HCl-SDS solution. The cells and the Tris-HCl-SDS solution were combined with the phenol/chloroform solution then shaken for 10 min at 7°C. The extract was centrifuged at 10,000 x g for 10 min, the supernatant removed and extracted twice with phenol/chloroform. At the third extraction a 1/10 volume of 2 M sodium acetate pH 4.7 was included in the phenol/chloroform. The RNA was precipitated by addition of a 2x volume of absolute ethanol and incubation at -20°C overnight. The RNA was pelleted by centrifugation, dried under vacuum, and then suspended in DEPC-treated ddH_2O. The 260/280 ratio was not lower than 1.7 for any of the samples used for analysis.

Total RNA (20 μg/lane) was loaded onto 1% agarose formaldehyde gels according to a modification of the Maniatis (1982) method. The gels (15 cm x 20 cm) were electrophoresed for 12 hr at 40v, then were removed and soaked for 20 min in 10x SSC buffer (1x SSC is 0.15 M NaCl; 0.15 M sodium citrate), and directly blotted to nitrocellulose by diffusion overnight. The blots were

then removed and baked in a vacuum oven at 80°C for 2 hr. The blots were prehybridized in 50% formamide, 1x Denhardt's solution, 25 μg/ml poly A, 6x SSPE, and 0.1% SDS for 24 hr at 37°C. The prehybridization buffer was removed and replaced with hybridization buffer of the same composition with the addition of the radio-labelled probe as indicated in the figure legends, then hybridized for 72 hr at 37°C. The blots were removed and rinsed in 2x SSC containing 0.5% SDS, washed twice for 15 min each in 2x SSC containing 0.1% SDS at room temperature, followed by 2, 3 hr washes in 0.1 SSC containing 0.1% SDS at 55°C. The blots were dried at room temperature prior to autoradiography at -70°C with intensifying screens.

2.3 Results

2.3.1 The Relationship of the Two Forms of Glycogen Phosphorylase During Development

Figure 2 shows that glycogen phosphorylase *b* activity was greatest in amoebae and then decreased during subsequent development, while phosphorylase *a* activity was absent in amoebae and then increased during subsequent development. The increase in phosphorylase *a* activity during late development has been observed by previous researchers (Firtel and Bonner 1972; Jones and Wright 1970; Thomas and Wright 1976a & b; Higgins and Dahmus 1982).

A western blot of extracts from several stages of development was immunostained with antibodies prepared to the two forms of the enzyme (Figure 3). Antiserum against the *b* form revealed a heavily stained protein at 92 kd throughout the time course of development, as well as a faintly staining protein at 104 kd in the later stages. Lower molecular weight proteins were sometimes stained with the anti-b serum and were found by peptide mapping to be degradation products of the 92 kd protein (not shown). Antiserum against the *a* form of the enzyme stained the 104 kd protein in the later stages of development but did not stain the 92 kd band. Staining of the 92 kd protein by anti-b antibody, and staining of only the 104 kd protein by anti-a antibody, suggested that the 104 kd protein represents the *a* enzyme while the 92 kd protein represents the *b* enzyme. This was substantiated by separating the two forms of the enzyme by ion-exchange chromatography then immunostaining western blots of each fraction (data not shown). Fractions containing *b* activity showed a 92 kd band while fractions containing *a* activity showed a 104 kd band. The difference in the apparent molecular weight of the enzyme on SDS gels could be the result of synthesis of the 104 kd peptide or phosphorylation of the 92 kd peptide. A shift in molecular weight has been observed in several proteins upon their phosphorylation by a protein kinase

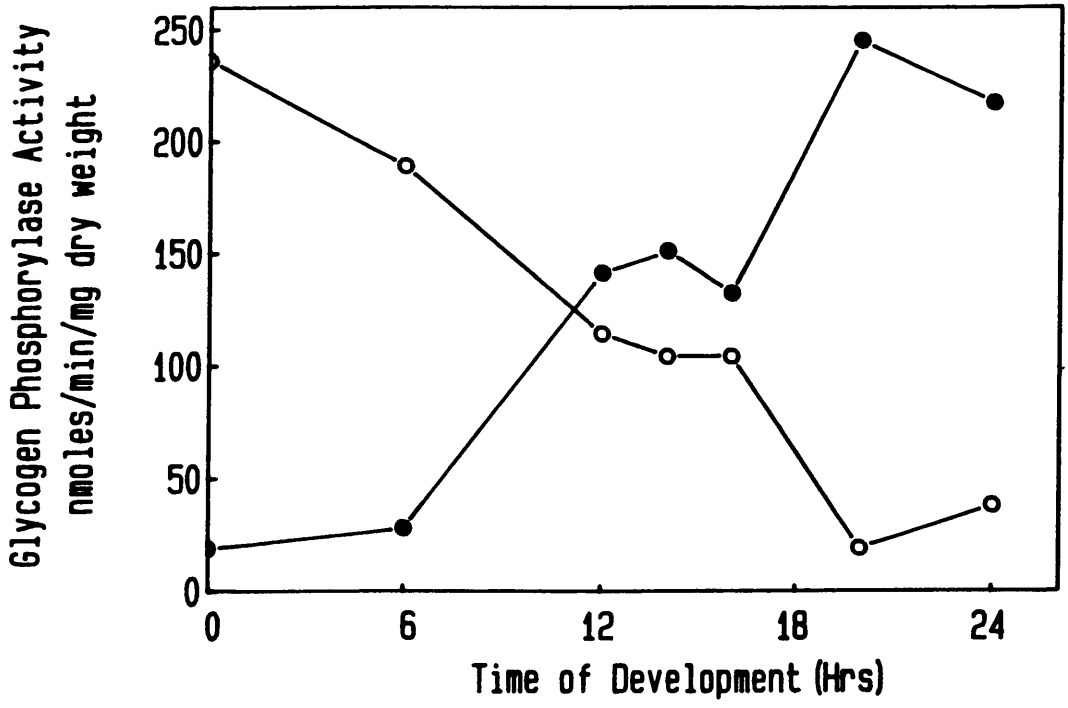

Figure 2. **Glycogen Phosphorylase Activity During Development on Filters:** Cells were allowed to develop on Gelman filters until harvested at progressive stages of development as described in methods. Glycogen phosphorylase activity was assayed in the presence of 5'AMP or in the absence of 5'AMP at the indicated times of development. Glycogen phosphorylase *b* activity (○) was present early in development and subsequently decreased during the remainder of development. Glycogen phosphorylase *a* activity (●) begins to appear at aggregation as indicated by decreasing dependency on 5'AMP for activity. Phosphorylase *b* activity was determined by substraction of activity measured in the absence of 5'AMP (phosphorylase *a* activity) form the total activity determined in the presence of 5'AMP.

(Dahmus 1981). Thus, the observed shift from *b* to *a* activity during development could be the result of phosphorylation of the 92 kd protein. Likewise, the 92 kd protein observed in late developmental stages (Figure 3A) could represent enzyme that has been dephosphorylated during sample preparation. Our lab is currently attempting to resolve this question.

2.3.2 Perturbation of Cells in Liquid Culture by cAMP and the Effect on the Two Forms of Glycogen Phosphorylase

Because the literature has described a 5′AMP **independent** phosphorylase activity that was induced by cAMP in shaking cultures (Takemoto et al. 1978; Okamoto & Takeuchi 1976), we decided to determine whether the 5′AMP **dependent** form was regulated by cAMP in shaking culture as well. Aggregation-competent cells were removed from non-nutrient agar plates as described in methods and assayed for phosphorylase activity in the presence and absence of 5′AMP. The phosphorylase activity in these aggregation-competent cell extracts was completely dependent on 5′AMP. When these cells were dispersed into Buffer B and shaken in the presence of 500 μM cAMP, the phosphorylase *b* activity decreased, until no *b* activity was detectable at 8 hr (Figure 4). In the absence of cAMP, the phosphorylase *b* activity remained constant throughout the incubation. In the presence of cAMP, phosphorylase *a* activity increased until at 8 hr the *a* activity was equal to the phosphorylase *b* activity of cells shaken in the absence of cAMP. Phosphorylase *a* activity was not detectable in extracts from cells shaken for 10 hr in the absence of cAMP. In Figure 5, we show the averaged results of three experiments plotted as the percent of the maximum activity that each form represented during the time the cells were exposed to cAMP. As the activity of the *b* form decreased, the activity of the *a* form increased in a corresponding manner. Total phosphorylase activity remained constant throughout the period of shaking. A similar situation is frequently observed in mammalian systems where phosphorylation of phosphorylase *b* results in the formation of phosphorylase *a* (Fisher and Krebs 1955; Cohen 1978). However, this

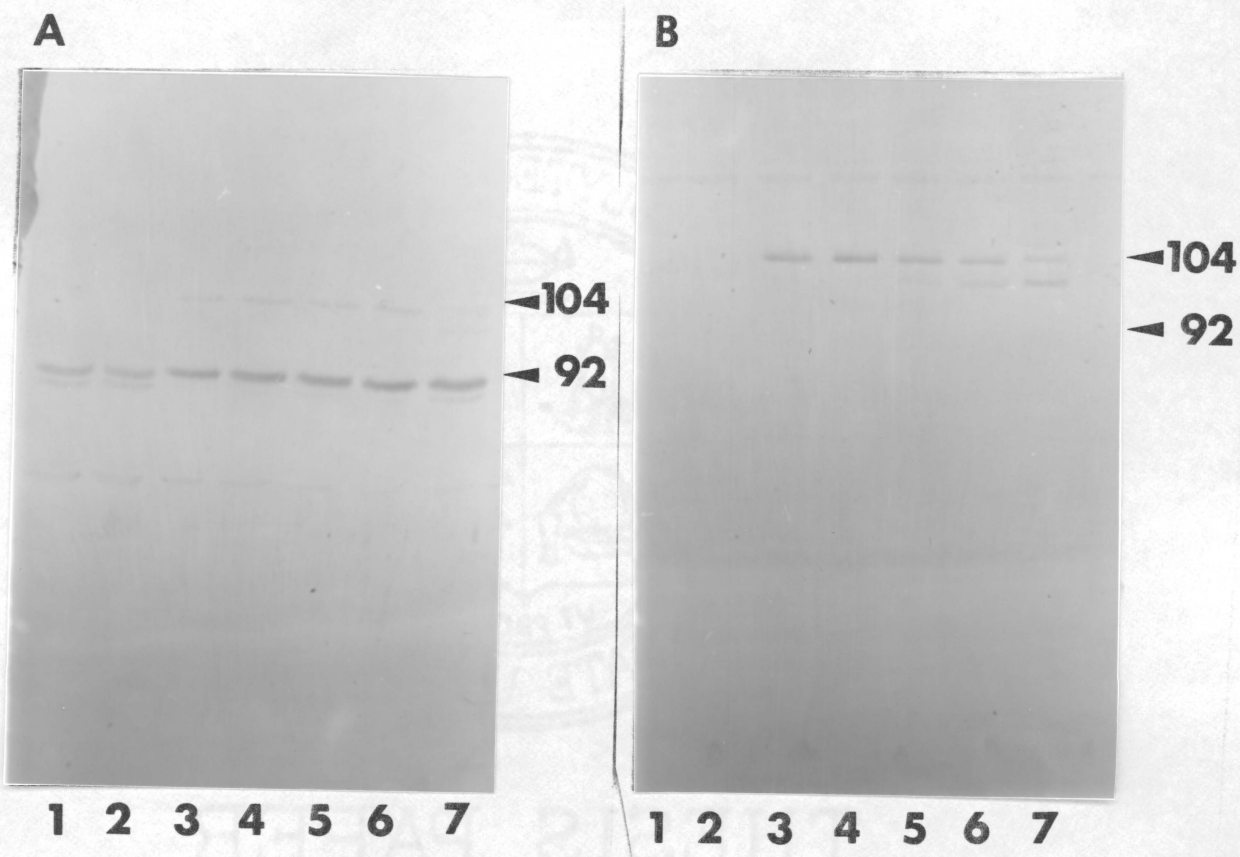

Figure 3. Western Blot of Glycogen Phosphorylase Activity During Development on Filters: SDS-PAGE samples from cell extracts containing glycogen phosphorylase activities at the developmental stages indicated in Figure 2, were electrophoresed and western blotted as described in methods. Replicate samples were run and the western blot cut in half. One half was stained with anti-b antiserum (A) and the other half stained with anti-a antiserum (B). The lanes for both blots were--**Lane 1**-- logarithmically growing Amoeba; **Lane 2**--6 hr rippling amoebae **Lane 3**--Aggregation; **Lane 4**--Early slug; **Lane 5**--Migrating slugs; **Lane 6**--Early culmination; **Lane 7**--Late culmination.

phosphorylation event in mammals occurs within minutes of exposure to hormones such as epinephrine rather than the several hours required in *Dictyostelium.*

We also followed the apparent molecular weight of phosphorylase under these conditions by preparing SDS-PAGE samples at 4 hr and 8 hr of shaking aggregation-competent cells in the presence and absence of cAMP. These samples were electrophoresed and western blotted as described in methods, then stained with anti-b serum (Figure 6A) or with anti-a serum (Figure 6B). The anti-b antibody stained a 92 kd protein in samples from cells shaken either in the presence or absence of cAMP (Figure 6A, Lanes 1-5). We have previously shown (Cloutier and Rutherford 1987) that the purified *Dictyostelium* phosphorylase *b*, has a molecular weight of 92 kd, thus indicating that the 92 kd protein shown in Figure 6 represents the phosphorylase *b* form. In addition, the rabbit phosphorylase *b* standard also has a similar subunit molecular weight of 94 kd. When extracts of cells that have been shaken in presence of cAMP were analyezed by SDS-PAGE, an additional band at 104 kd was weakly stained by anti-b antibody (Figure 6A, Lanes 2 and 4). Phosphorylase *a* activity represented 30%-50% of the total activity in cells shaken for 4 hr and 100% for 8 hr cells (Figure 5). When these same blots were stained with the anti-a serum only the 104 kd protein was stained and only in cells shaken in the presence of cAMP (Figure 6B, Lanes 2 and 4). No proteins were stained by the anti-a antibody in samples from cells shaken in the absence of cAMP (Figure 6B, Lanes 1 and 3). Extended staining of the western blot in Figure 6B for 2 hr did not reveal any proteins in addition to the 104 kd protein. Thus, perturbation of undifferentiated cells by cAMP in liquid culture regulates the activity and subunit molecular weight of the enzyme in a manner that is identical to cells developing normally on a solid substrate and in the absence of exogenous cAMP.

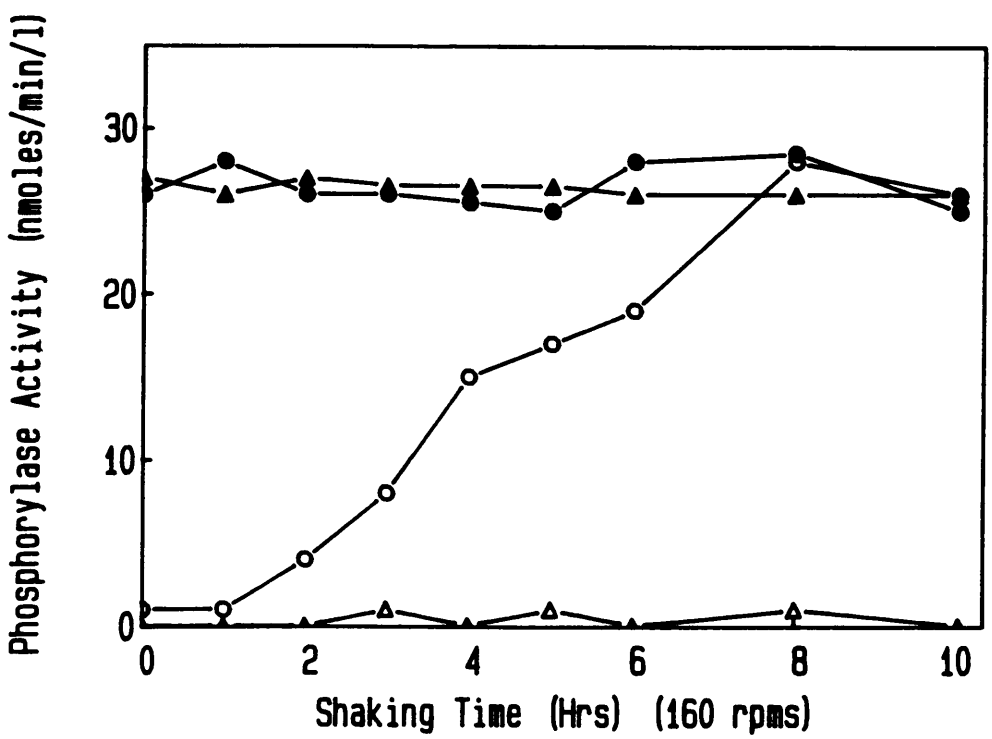

Figure 4. **Cyclic AMP Induction of 5'AMP Independent Activity in Shaking Culture:** Aggregation-competent cells were shaken in Buffer B in the presence (circles) and absence (triangles) of 500 μM cAMP. Cyclic AMP was added at the 0 time point and at the 4 hr time point. Phosphorylase activity was measured in the presence of the allosteric activator, 5'AMP (●, ▲) and in its absence (○, △).

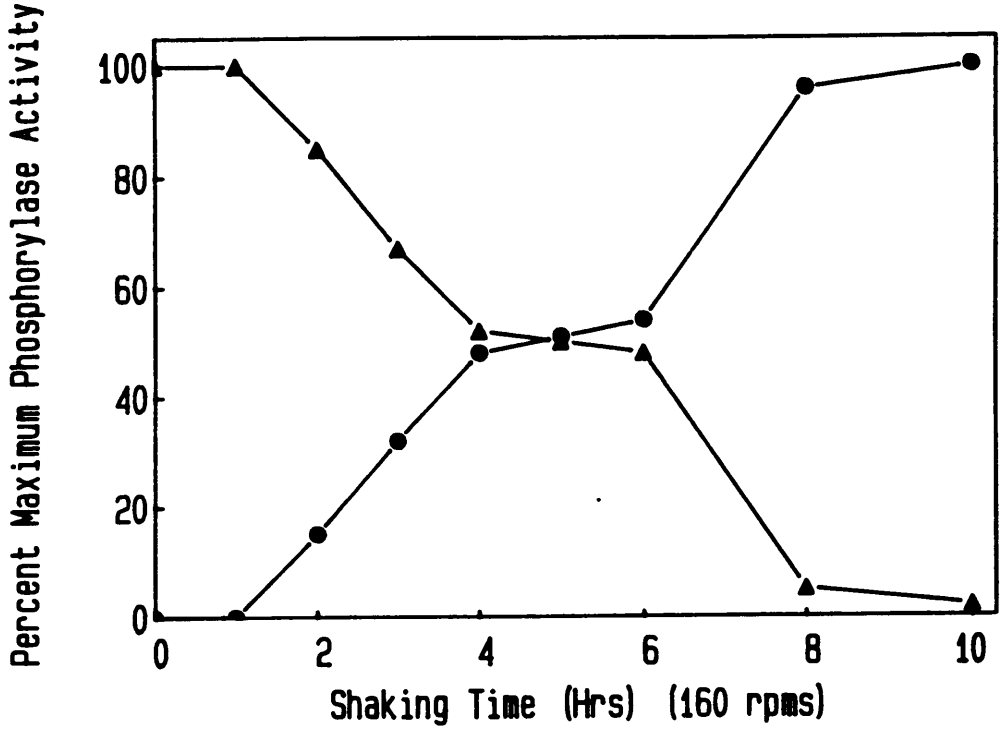

Figure 5. **Ratio of the Two Forms of Phosphorylase from Cells Exposed to Extracellular cAMP:** This figure illustrates the percent of the total activity each form of the enzyme represented as determined by dependency on 5′AMP for activity during the shaking experiment. The dependent activity (▲) is the *b* form and the nondependent activity (●) is the *a* form. Each point represents the average of three experiments.

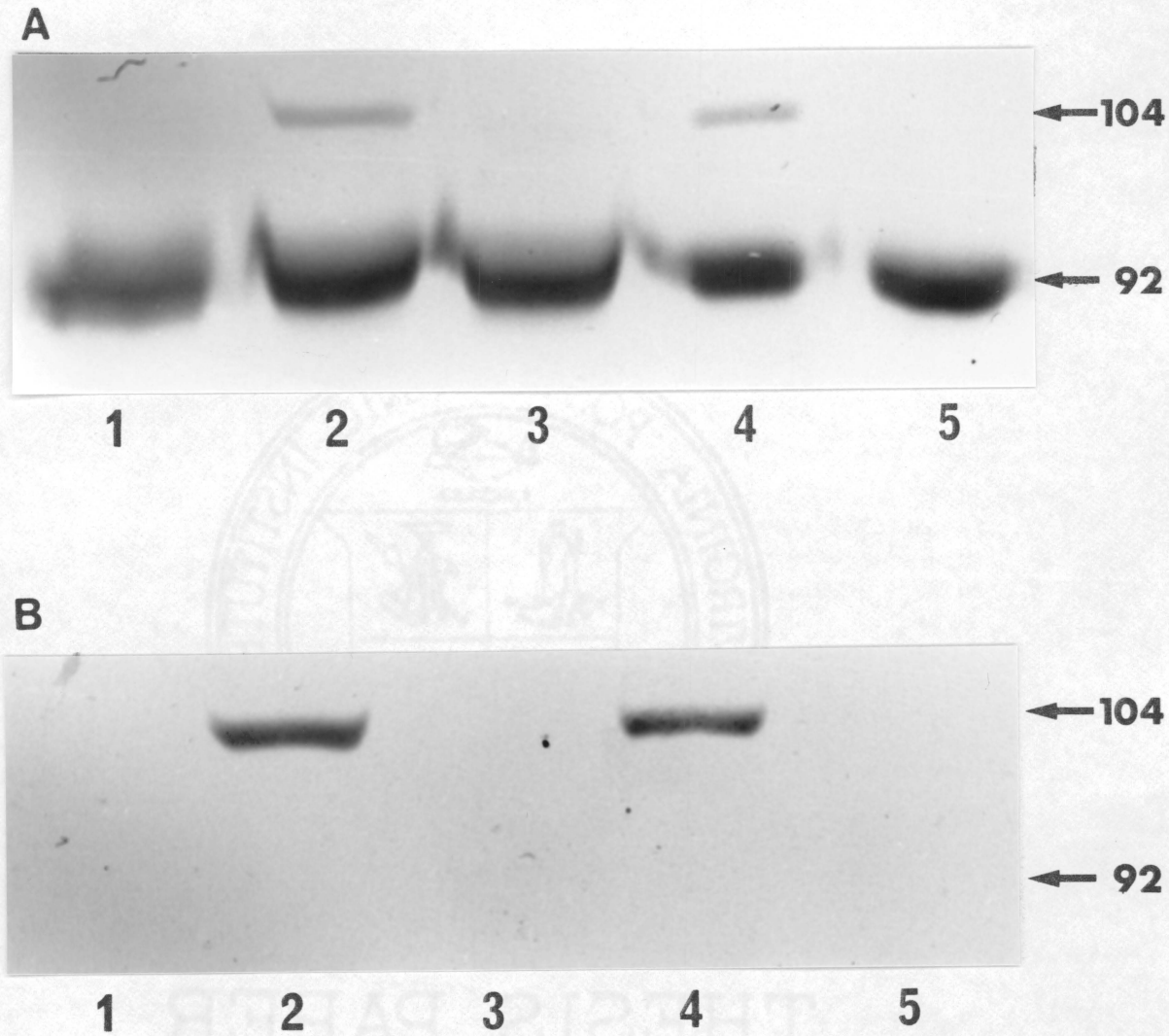

Figure 6. Western Blot of Cells Exposed to Extracellular cAMP: Duplicate sets of samples from 4 hr and 8 hr exposure to added cAMP were run on 6% SDS-PAGE, blotted onto nitrocellulose; the nitrocellulose was cut in half, one-half stained with anti-b serum (A) and the other half stained with anti-a serum (B). **A. Lane 1**--4 hr shaking, no treatment, **Lane 2**--4 hr shaking, 500 μM cAMP, **Lane 3**---8 hr shaking, no treatment, **Lane 4**--8 hr, 500 μM cAMP, **Lane 5**--aggregation-competent cells (not shaken). The arrows indicate a 92 kd rabbit phosphorylase *b* standard and a 104 kd phosphorylase *a* standard. The only protein detected by the anti-a serum was a 104 kd band in the (+) cAMP lanes. The anti-a serum was made to purified phosphorylase *a* which migrated at 92 kd on SDS-PAGE.

2.3.3 The Effect of cAMP Analogs on the Regulation of the Two Forms of Glycogen Phosphorylase

Recent reports have tried to identify the pathway for transcriptional regulation in *Dictyostelium* by using several cAMP analogs that have differential affinities for the membrane-bound cAMP receptor, the cytoplasmic cAMP-dependent protein kinase (cAMPdPK), adenylate cyclase, and cAMP phosphodiesterase (Oyama & Blumberg 1986a,b,c; Haribabu & Dottin 1986; Schaap & van Driel 1985). These reports indicate that regulation of transcriptional activity may be mediated through the cAMP receptor. We investigated the effects of the cAMP analogs used in these reports, as well as additional treatments, on the regulation of the two forms of glycogen phosphorylase. The regulation of previously characterized cAMP-regulated mRNAs by the cAMP analogs served as controls. The cDNAs (PL3,D11, and PL3) were graciously provided by Daphne Blumberg for this investigation. PL3 and D11 are induced by the addition of cAMP to cells in shaking culture. PL3 is a prespore specific mRNA of 2.3 kb, belonging to class II or late prespore mRNAs. D11 is enriched in prestalk cells and belongs to the early class of mRNAs. D3 is a mRNA produced in vegetatively growing cells; it is repressed by the addition of cAMP to shaking cells (Oyama & Blumberg 1986a,b,c; Haribabu & Dottin 1986).

Selection of the cAMP analogs was based upon their affinities for the cAMP receptor and the regulatory subunit of the cAMPdPK. The analog 2'd-cAMP has a high affinity for the cAMP receptor but a relatively low affinity for the cAMPdPK. 2'd-cAMP induces both PL3 and D11 while also repressing D3 at levels similar to those at which cAMP causes induction and repression of these genes (Oyama and Blumberg 1986; Haribabu and Dottin 1986; Mann and Firtel 1987). 8Br-cAMP and Bt-cAMP have low affinities for the cAMP receptor but high affinities for the cAMPdPK. Bt_2-cAMP is membrane permeable in higher eucaryotes but its permeability has not been demonstrated in *Dictyostelium*. Bt_2-cAMP is used in higher eucaryotic studies to mimic the second messenger effects of cAMP while by-passing the signal transduction mechanism. It has little affinity for the cAMP receptor but high affinity for cAMPdPK. cGMP and cIMP were used to test

the specificity of the induction for cAMP and its analogs. Neither has high affinities for the cAMP receptor nor the cAMPdPK. PMA was selected since its mechanism of action involves neither the receptor nor the cAMPdPK; instead, it activates protein kinase C in some organisms. Glycogen phosphorylase activity was measured in shaking cultures to which the cAMP analogs listed in Table 1 had been added. All components tested were added to 500 μM final concentration except for PMA which was added to 100 ng/ml. In agreement with a previous report (Schaap and van Driel 1985), 2'd-cAMP was approximately equal to authentic cAMP in promoting the appearance of phosphorylase *a* activity. At a 500 μM concentration, 8Br-cAMP and Bt_2-cAMP were able to promote a 90% conversion of *b* to *a* activity in the 8 hr time period. At concentrations below 500 μM, 8Br-cAMP and Bt_2-cAMP were unable to produce a conversion of phosphorylase *b* to *a*. Schaap and van Driel (1985) found phosphorylase *a* to appear only in response to a 4-fold greater concentration of 8Br-cAMP than that required for cAMP. Oyama and Blumberg (1986) found that a 500 μM concentration of Bt_2-cAMP did not induce or repress the activity of any of the cAMP inducible genes. Table 1 shows that neither cGMP nor cIMP at 500 μM or 1 mM caused conversion of the phosphorylase activities. PMA at 100 ng/ml did not induce conversion of the enzyme forms. It has been reported that prolonged exposure of higher eucaryotes to PMA resulted in down regulation of protein kinase C (Collins & Rozengurt 1982). We, therefore, took samples at 10 min and 30 min after aggregation-competent cells were exposed to PMA, but again, no conversion of phosphorylase activities was observed. This lack of an effect may be due to the inability of PMA to enter the cell and activate protein kinase C in *Dictyostelium.*

In order to determine the effect of extracellular cAMP and the analogs on the subunit molecular weight of phosphorylase, a western blot of selected samples from Table 1 was stained with a mixture of anti-a and anti-b antiserum. The 104 kd protein, indicative of the phosphorylase *a* activity, was observed in samples from cells shaken in the presence of cAMP, 2'd-cAMP, Bt_2-cAMP, and 8Br-cAMP for 8 hr (Figure 7, Lanes 4, 6, 9, and 11, respectively). The cAMP analogs, 8Br-cAMP and Bt_2-cAMP, which did not induce phosphorylase *a* activity as well as cAMP or 2'd-cAMP, showed a faint 104 kd band after shaking for 4 hr (Figure 7, Lanes 8 and 10), whereas, cAMP and 2'd-cAMP always gave a strongly staining 104 kd protein after 4 hr (Figure

Table 1. The Effect of cAMP and Its Analogs on the Percentage of Phosphorylase "a" in Shaking Aggregation-Competent Cells[1]

Treatment	Percentage of Total Phosphorylase Activity Due to the "a" form[2]	
	4 HR Shaking	8 HR Shaking
No Treatment	0	2
No Treatment in Glucose/Albumin Media[3]	0	0
cAMP	37	102
cAMP in Glucose/Albumin Media	0	0
2'deoxy-cAMP	43	100
8-Bromo cAMP	21	91
Dibutyryl cAMP	25	88
Monobutyryl cAMP	15	83
cGMP	0	0
cIMP	0	8
PMA	0	0

[1]Analogs were chosen based upon their affinities for the cAMP receptor (cAMP-R) and the cAMP-dependent Protein Kinase (cAMPdPK). The analog, 2'deoxy-cAMP, has a high affinity for the cAMP-R while the others have higher affinities for the cAMPdPK than the cAMP-R. PMA is Phorbol-myristate-acetate, a known activator of Protein Kinase C. All analogs were of the highest purity available from Sigma. All analog concentrations were 500 μM. PMA was added to a concentration of 100 ng/30 ml.

[2]The Phosphorylase Total Activity remained reasonably constant throughout the experiment. The activity from Aggregation-Competent cells assayed in the presence of 5'AMP was set equal to 100% and the activity assayed in the absence of 5'AMP was set equal to 0%. The percentage of the total activity due to the "a" form was determined by the amount of increase in the activity measured in the absence of 5'AMP.

[3]Glucose-Albumin medium contains 5% glucose and 2% albumin in Mes-LPS pH 6.5. Oyama and Blumberg had found his medium to inhibit adenylate cylase activity.

7, Lanes 4 and 6). Only the 92 kd protein was stained in samples from cells shaken in the presence of cGMP or PMA (Figure 7, Lanes 12-15).

The above results suggest that an intracellular increase in cAMP is necessary for conversion of phosphorylase *b* to *a* actvity and for induction of the 104 kd *a* protein. Both 8Br-cAMP and Bt_2-cAMP, which possess high affinities for the cAMPdPk, were able to convert *b* to *a* activity and induce the 104 kd protein. The same effects were produced by 2'd-cAMP, which shows a high affinity for the receptor. This result does not show, however, that the cAMP receptor can regulate the phosphorylase by a mechanism that bypasses an increase in intracellular cAMP, because under the conditions of the experiment, binding of the receptor by 2'd-cAMP results in activation of adenylate cyclase and a corresponding increase in intracellular cAMP levels.

Under the same conditions as shown in Figures 6 and 7, we followed the expression of several genes that are known to be regulated by cAMP, and compared the results to the effect of cAMP and the analogs on the distribution of the *a* and *b* forms of phosphorylase. Total RNA was extracted from cells treated in shaking culture with 500 μM cAMP, 2'd-cAMP, 8-Br-cAMP, and Bt_2-cAMP. Samples were prepared as described in methods and 20 μg was applied to each lane for electrophoresis, then transferred to nitrocellulose. The blots were hybridized to nick-translated cDNA probes followed by autoradiography. The prespore specific mRNA, PL3, and the prestalk specific mRNA, D11, were induced when cells were exposed to cAMP, 2'd-cAMP, 8Br-cAMP, and Bt_2-cAMP (Figure 8, Lanes 4-7 and 9-12). D3, the growth specific mRNA, was repressed in the presence of these same analogs (Figure 8, Lanes 4-7 and 9-12). The intensity of the bands and the overall results obtained with cAMP and 2'd-cAMP were comparable to those obtained by Oyama and Blumberg (1986a,b,c 1987) under similar conditions. However, Oyama and Blumberg found that, 500 μM Bt_2cAMP did not induce PL3 and D11 nor did it repress D3. There are two possible reasons for differences in gene regulation by Bt_2cAMP under our conditions and those of Oyama and Blumberg--(1) We used aggregation-competent cells whereas Oyama and Blumberg used aggregation stage or later stages for their experiments. We used aggregation-competent cells because we had originally observed the cAMP induction of phosphorylase *b* to *a* at this stage of development; (2) Oyama and Blumberg extracted RNA after 2.5 hr exposure to cAMP or cAMP analogs;

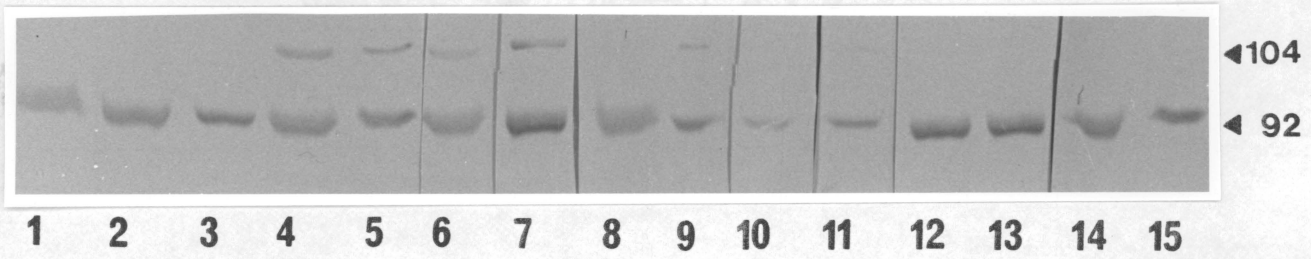

Figure 7. Western Blot Showing the Effect of cAMP Analogs on the Two Forms of Phosphorylase: Samples from 4 hr and 8 hr shaking were run on 6% SDS-PAGE, blotted onto nitrocellulose and stained with a mixture of anti-a and anti-b antibodies. Lane 1--aggregation-competent cells prior to shaking; Lanes 2, 3--4 hr and 8 hr,shaking, respectively, no treatment; Lanes 4, 5--4 hr and 8 hr, 500 μM cAMP;. Lanes 6, 7--4 hr and 8 hr, 2′deoxy cAMP; Lanes 8, 9--4 hr and 8 hr, 8-Br-cAMP; Lanes 10, 11--4 hr and 8 hr, Bt_2-cAMP; Lanes 12, 13--4 hr and 8 hr, PMA; Lanes 14, 15--4 hr and 8 hr, cGMP. The arrows indicate the position of 92 kd and 104 kd standards. Only those samples showing phosphorylase *a* activity exhibited the 104 kd band. Although the 104 kd band in Lane 11 may appear faint in this photograph, the band was clearly visible in the freshly stained Western blot.

we extracted RNA after 4 hr and 8 hr exposures to cAMP or cAMP analogs. We used such long incubation periods because conversion from phosphorylase *b* to *a* activity required at least 8 hr of exposure to cAMP for complete conversion to the *a* form (Figure 4).

2.3.4 The Effect of Glucose/Albumin Medium on the Regulation of the Two Forms in Shaking Culture

Oyama and Blumberg (1986a,b,c) had previously reported the use of glucose/albumin medium to inhibit adenylate cyclase activity and thus prevent an intracellular increase in cAMP in response to extracellular cAMP. It was reasoned by these authors that if the cAMP dependent genes were induced by extracellular cAMP under conditions in which adenylate cyclase was not active, this would be evidence against the involvement of the cAMPdPK in gene regulation. The regulation of the gene activity would result instead by other mechanisms, perhaps from the interaction of extracellular cAMP and the membrane-bound cAMP receptor. These authors showed that if aggregation stage cells were slowly shaken in glucose/albumin medium (160 rpm) containing cAMP, the genes were induced without any apparent intracellular increase of cAMP, thus providing evidence against the involvement of cAMPdPK in gene regulation.

We tested the cAMP induction of phosphorylase *a* activity in cells that were shaken in the glucose/albumin medium. However, instead of using aggregation stage cells as described by Oyama and Blumberg (1986a,b,c) we used aggregation-competent cells, a stage several hours earlier in development. We chose this stage of development because Schaap and van Driel (1985) showed the induction of phosphorylase *a* activity and the effect of several cAMP analogs on phosphorylase activity under these conditions. By definition, aggregation-competent cells are able to aggregate within 30 min of plating onto a nonnutrient substrate. Aggregation-competent cells also have all the necessary components for differentiation (Loomis, 1982). When these cells were exposed to cAMP in the glucose/albumin medium, glycogen phosphorylase *b* was not converted to

Figure 8. The Effect of cAMP and its Analogs on Three Developmentally Regulated mRNAs in Shaking Culture: Total RNA was purified from a shaking experiment which exhibited conversion of the two forms of phosphorylase. An autoradiogram of a northern blot of these mRNAs hybridized to the three cDNA probes is shown here. PL3 and D11 were induced by cAMP and the analogs while D3 was repressed under these conditions. Lane 1--amoebae, Lane 2--aggregation-competent cells; Lane 3--4 hr, no treatment; Lane 4--4 hr, cAMP; Lane 5--4 hr, 2′dcAMP; Lane 6--4 hr, 8Br-cAMP; Lane 7--4 hr, Bt_2-cAMP; Lane 8--8 hr, no treatment; Lane 9--8 hr, cAMP; Lane 10--8 hr, 2′d-cAMP; Lane 11--8 hr, 8Br-cAMP; Lane 12--8 hr, Bt_2-cAMP. cAMP and its analogs were added to final concentrations of 500 μM as described in methods.

phosphorylase *a* (Figure 9, Lanes 6-9). However, control cultures containing 500 μM cAMP in Buffer B showed the usual conversion of phosphorylase *b* to *a* (Figure 9, Lanes 4 and 5) as indicated by the staining of the 104 kd protein and conversion of enzyme activity. In order to test if the glucose/albumin medium was capable of influencing the effect of cAMP on gene activity in these aggregation-competent cells, we performed Northern analysis of the PL3, D11, and D3 mRNAs (Figure 10). When cells were exposed to a cAMP-glucose/albumin medium there was no effect on the mRNA levels for the three genes (Figure 10, Lane 1) as compared to cells treated with glucose/albumin in the absence of cAMP. In either case D3 was not repressed, nor was D11 or PL3 induced. However, a cAMP effect was observed in control cells shaken in Buffer B. Figure 10 (Lane 2) shows that in Buffer B, cAMP represses D3 and induces D11 and PL3. Substitution of the analog Bt_2-cAMP showed identical results to authentic cAMP, that is, no effect in the glucose/albumin media (Figure 10, Lane 3) and repression of D3 and induction of D11 and PL3 in Buffer B (Figure 10, Lane 4).

These results show that the glucose-albumin medium eliminates the effect of added extracellular cAMP since in the absence of this medium perturbation by cAMP results in conversion of enzyme activity as well as regulation of gene activity. Because adenylate cyclase is inhibited under these conditions the results indicate that an intracellular increase in cAMP is required for the *b* to *a* conversion of phosphorylase. The results also show a difference in the regulation of gene activity according to the developmental stage of cells that are used for the shaking cultures.

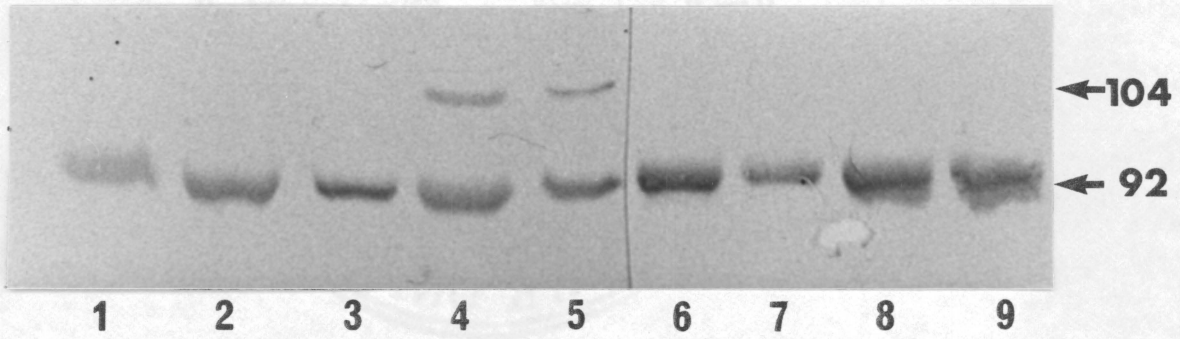

Figure 9. The Effect of Glucose/Albumin Medium on the cAMP Induction of the Two Forms of Phosphorylase in Shaking Culture: Glucose/albumin medium as shown by Oyama and Blumberg (1986) inhibits adenylate cyclase activity thus preventing an intracellular increase in cAMP during shaking. They were able to show that certain developmentally regulated mRNAs were induced by extracellular cAMP in this glucose/albumin medium, and therefore the gene induction did not require an adenylate cyclase produced increase in intracellular cAMP. However, as illustrated here, glycogen phosphorylase *a* did not appear in the presence of cAMP in glucose/albumin medium. **Lane 1**--aggregation-competent cells prior to shaking; **Lanes 2, 3**--4 hr and 8 hr, no treatment; **Lane 4, 5**--4 hr and 8 hr, 500 μM cAMP only; **Lane 6, 7**--4 hr and 8 hr, no treatment in glucose/albumin; **Lane 8, 9**--4 hr and 8 hr, 500 μM cAMP in glucose/albumin. The arrows indicate the position of 92 kd and 104 kd standards.

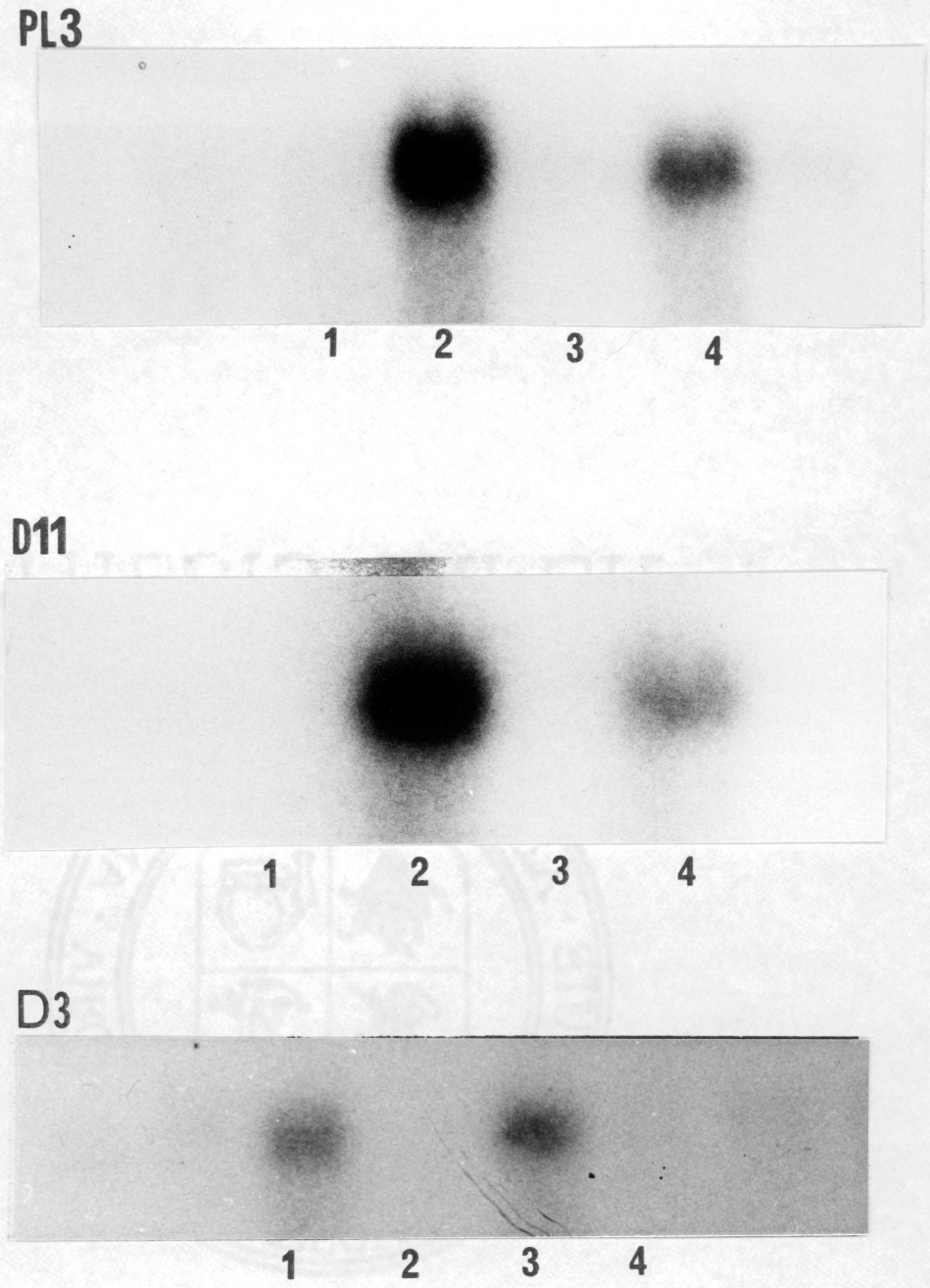

Figure 10. Effect of glucose/albumin medium on cAMP Induction of Gene Activity: Northern analysis was done as described in the legend to Figure 8. Cells were shaken for 8 hr in the presence or absence of 500 μM cAMP or 500 μM Bt_2-cAMP with either glucose/albumin medium or Buffer B as the shaking medium. **Lane 1**--500 μM cAMP in glucose/albumin; **Lane 2**--500 μM cAMP in Buffer B; **Lane 3**--500 μM Bt_2-cAMP in glucose/albumin; **Lane 4**--500 μM Bt_2-cAMP in Buffer B.

2.4 Discussion

We have demonstrated previously the existence of two glycogen phosphorylase activities that are comparable in physical and enzymatic properties to the two interconvertible forms observed in other eucaryotes (Rutherford and Cloutier 1986; Cloutier and Rutherford 1987). We have further shown here that these two forms of phosphorylase are opposingly regulated by cAMP in shaking culture. The activity of the *b* form decreased in response to cAMP in shaking culture, whereas, the phosphorylase *a* form increased. This same pattern of *b* to *a* conversion and the appearance of a 104 kd peptide occurs during normal development in which no external addition of cAMP is required. In future studies, we will test the hypothesis that the regulation of glycogen phosphorylase during development is regulated by an increase in intracellular levels of cAMP. In fact, we have shown earlier (Pahlic and Rutherford 1979) that both adenylate cyclase activity and intracellular levels of cAMP are nearly undetectable until 10 hr of development, then suddenly increase and remain high during the later stages. As shown in Figure 2, this increase in intracellular cAMP coincides with the phosphorylase *b* to *a* conversion. This temporal relationship of cAMP levels and phosphorylase regulation during the course of normal development as well as the effect on phosphorylase of cAMP perturbation of cells in liquid culture are strongly suggestive that cAMP regulates the enzyme *in vivo*. This pattern of response to cAMP could indicate the interconversion of two forms of a single enzyme or, alternatively, coordinate regulation of two separate genes. Current work in this laboratory is addressing this question.

The data presented here suggest that regulation of the two forms of glycogen phosphorylase is mediated by a mechanism that requires an intracellular increase in cAMP. Several reports in the literature have implicated the cell surface receptor as having a direct role in mediating cAMP regulation of gene expression, rather than a mechanism involving an intracellular increase in cAMP and a cAMPdPK (Oyama and Blumberg 1986a,b,c; Kimmel 1987; Haribabu & Dottin 1986; Schaap & van Driel 1985). However, the experimental conditions in these various laboratories have differed

in the developmental stage of the cells used, the incubation time during which cAMP and its analogs were applied and in the medium that was used. For example, Oyama and Blumberg (1986a,b,c) observed cAMP dependent gene regulation in cells that were shaken for only 2.5 hr in glucose/albumin media. We found no detectable glycogen phosphorylase *a* activity at 4 hr or 8 hr in cells that were shaken in the presence of cAMP; neither did we see gene regulation under our conditions.

There are also some difference in the literature regarding the regulation of glycogen phosphorylase *a* activity by cAMP. For example, Takeuchi and coworkers (Takeuchi & Sakai 1971; Takemoto et al 1978; Okamoto & Takeuchi 1976) followed phosphorylase *a* activity of aggregation-competent cells in a glucose/albumin medium similar to that used by Oyama and Blumberg (1986a,b,c). Okamoto et al. (1976) did not observe glycogen phosphorylase *a* activity until 15 hr shaking, with the peak of activity at 30 hr shaking, whrereas, we found the peak of *a* activity at 8 hr shaking under our conditions. Schaap and van Driel (1985) found that 8Br-cAMP could not induce phosphorylase *a* activity, while in our hands 8Br-cAMP did induce this activity; however, Schaap and van Driel exposed cells to pulses of 8Br-cAMP for 4 hr while we exposed cells to a continuous high level of the analog.

In our experiments gene regulation and phosphorylase induction exhibited identical requirements for intracellular cAMP, suggesting that both occur via the same pathway. However, given the differences between the results of similar experiments in several laboratories, the exact nature of this pathway remains unclear. In some cases (Oyama and Blumberg 1986a,b,c; Haribabu & Dottin 1985; Kimmel 1987; Mann & Firtel 1987; Gomer et al. 1986) the cAMP receptor is directly involved in the signal transduction mechanism based upon the ability of the cAMP analogs with high affinity for the cAMP receptor to induce gene activation. Further evidence in support of the involvement of a cAMP receptor in gene regulation is the recent report on the translocation of a membrane cAMP-binding protein to the nucleus (Kay et al. 1987). This binding protein has been characterized and is different from the cAMP binding protein that forms the regulatory subunit of cAMPdPK. The role of the known intracellular increases in cAMP during development of *Dictyostelium* and the resultant effect on cAMPdPk activation remain to be determined. Kimmel

(1987) has proposed that at least two mechanisms for cAMP regulation of gene expression are present in *Dictyostelium*, one which is dependent upon intracellular increases in cAMP. Of related interest, a cAMPdPK has been recently described in yeast (Behrens and Mazon 1988) that was released from the cell membrane upon binding of cAMP to the regulatory subunit of the kinase. Likewise, a cAMPdPK has recently been described in *Dictyostelium* that is membrane bound and phosphorylates the membrane-bound cAMP receptor (Meier and Klein 1988). Thus, membrane-bound kinases could provide yet another possible mechanism to mediate the extracellular cAMP signal in the absence of an intracellular increase in cAMP. Further investigation of glycogen phosphorylase regulation at the levels of enzyme activity as well as gene regulation will provide a useful model for the study of cAMP regulation of cellular differentiation. This enzyme system will provide one of the few systems in *Dictyostelium* where the product of the cAMP regulated gene is known and well characterized. Current research is focusing on the cloning of the phosphorylase gene(s) and investigation of its possible regulation by cAMP.

3.0 Endogenous Phosphorylation of Glycogen Phosphorylase b in Dictyostelium

Abstract

The slime mold, *Dictyostelium discoideum*, has two forms of the enzyme glycogen phosphorylase. The inactive phosphorylase *b* form requires 5'AMP for activity and is present in early development. The active phosphorylase *a* form is 5'AMP independent and occurs during later development. In the presence of exogenously added Mn^{2+} and ATP, the phosphorylase *b* shows apparent conversion into a 5'AMP independent form as measured by enzyme activity. In addition, Mn^{2+} and ATP also support an *in vitro* phosphorylation of the 92 kd phosphorylase *b* subunit. A protein kinase was found to overlap with phosphorylase *b* activity when eluted from a DE-52 anion exchange column of amoebal extracts. Endogenous phosphorylation of phosphorylase *b* was greatest in this region of overlap. A comparison of the phosphorylation requirements of this protein kinase for casein, as a substrate, and the *in vitro* phosphorylation of phosphorylase *b* showed similar cation requirements and pH optima. *In vivo* studies have also shown phosphorylation of phosphorylase *b* indicating a possible physiological significance for its phosphorylation.

3.2 *Introduction*

Earlier studies on the glycogen phosphorylase in *Dictyostelium discoideum* reported that the enzyme was developmentally regulated (Firtel & Bonner 1972; Gustafson & Wright 1972; Jones & Wright 1970; Marshall et al. 1970; Rutherford & Harris 1976; Wright et al. 1968; Higgins & Dahmus 1982). These studies found little or no phosphorylase activity during early development, followed by a peak of 5'AMP independent activity during late development. We have recently reported on the purification and characterization of two forms of glycogen phosphorylase, a 5'AMP dependent *b* form and a 5'AMP independent *a* form (Cloutier & Rutherford 1987). With our discovery of an inactive 5'AMP dependent form of *Dictyostelium* phosphorylase during early development, questions on the relationship between the two enzyme forms arose. Specifically, we wished to test the hypothesis that the active 5'AMP independent form of the phosphorylase (phosphorylase *a*) resulted from a conversion of the inactive 5'AMP dependent form (phosphorylase *b*) via a cAMP mediated phosphorylation. Although, regulation of phosphorylase activity by phosphorylation-dephosphorylation is a common event in systems that store glycogen as an energy reserve, the unique role played by phosphorylase in *Dictyostelium* development may necessitate that it is regulated in a different manner. It has been established in the literature that the glucose units produced by the phosphorylase reaction are utilized during terminal differentiation for synthesis of a cellulosic stalk and spore coat, rather than for energy production (Gustafson & Wright 1972; Marshall et al. 1970; Wright et al. 1968). Thus, *Dictyostelium* glycogen degradation is an integral part of a programmed developmental cycle rather than a method of energy production. We are, therefore, considering the possibility that the two forms of *Dictyostelium* glycogen phosphorylase represent separate gene products. This hypothesis is lent support by the fact that partially purified forms of the enzyme are immunologically distinct and have different subunit molecular weights as detected by immunoblotting. We showed earlier that the purified phosphorylase *b* has a subunit molecular weight of 92 kd, whereas the partially purified *a* enzyme has a subunit molecular weight of 104 kd. (Naranan et al. 1988). In this paper we describe the phosphorylation of the phosphorylase *b in vitro*

and *in vivo*. An endogenous protein kinase that catalyzes this phosphorylation was observed. The phosphorylation occurs under conditions that result in the apparent conversion of phosphorylase *b* activity into a 5'AMP independent activity.

3.2 Materials and Methods

3.2.1 Materials

[γ^{32}P] ATP (25-30 Ci/mmol) was purchased from NEN. Nitrocellulose was purchased from Fisher Scientific, Pittsburg, PA. DE52 cellulose from Whatman Inc., Clifton, NJ. and Protein A (Pansorbin cells) from Calbiochem Corp., San Diego, CA. Other reageants were purchased from Sigma Chemical Co., St. Louis, MO.

3.2.2 Methods

3.2.2.1 Cell Harvest and Development.

Dictyostelium discoideum (AX3) was grown in liquid HL5 medium on a rotary shaker as previously described (Sussman & Sussman 1967) The resulting amoebae were harvested by centrifugation in a continous flow rotor, then were washed and resuspended in 50 mM Tris-HCl pH 7.5 containing 2 mM benzamidine, 2 mM 2-mercaptoethanol and 0.02 % sodium azide. (Buffer A). When differentiated cells were required, the amoebae were washed free of media, diluted 1:3 (weight:volume) in 7 mM N-morpholinoethanesulfonic acid, pH 6.5, containing 20 mM KCl and 5 mM $MgSO_4$ (Buffer B). The washed cells were plated onto non-nutrient agar or Gelman GN-6 membrane filters supported by Gelman absorbent pads (both membranes and pads were pre-soaked for 30 min in Buffer B). At the required developmental stage, cells were harvested off the non-nutrient agar in buffer A + 20% sucrose, and lysed by sonication. Cells developing on the Gelman membranes were frozen at -70°C. Upon thawing, 0.5 ml of Buffer A was applied to each pad. The lysates were scraped from the membranes and centrifuged at 10,000 x g for 6 min. The resulting

supernatants were assayed for glycogen phosphorylase activity and prepared for SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.2.2 Preparation of Cell Lysates

The washed amoebae (60-100 g wet weight) were suspended in 3 volumes of cold buffer A containing 20% sucrose and 1 mg/l of the protease inhibitor, antipain. The cells were lysed by subjection to five 45 second exposures to a 2 cm probe of a sonic cell disrupter (Model 300, Fisher). After the lysate was centrifuged at 100,000 x g for 1 hr, neutralized protamine sulfate was added at a final concentration of 3 mg/g wet wt. of cells. This extract was then centrifuged at 100,000 x g for 45 min and the resulting supernatant was used for column chromatography. For the experiments shown in Figures 11, 12, and 14, cells at the migrating slug stage were harvested from the Gelman membranes and lysed as explained earlier. For the experiments shown in Figures 15 and 16, phosphorylase *b* and casein kinase activities obtained by DE-52 cellulose chromatography were used. The appropriate DE-52 column fractions were concentrated and washed with Buffer B followed by buffer exchange on a Sephadex PD-10 column in Buffer A at pH 8.2 or 50 mM phosphate buffer pH 6.8 (Buffer C)

3.2.2.3 Column Chromatography

The 100,000 x g supernatant from the amoeba stage was batch treated with 0.1 volume of DE-52 cellulose resin that had been equilibrated in Buffer A. After stirring 10 min, the resin was settled by centrifugation. The resin was then placed in a column and bound proteins were eluted with an 8 hr linear 0-0.25 M KCl gradient in buffer A containing 10% glycerol. Phosphorylase *b* and the kinase activities eluted at 150-180 mM KCl, whereas phosphorylase *a* activity eluted at 50 mM KCl. Column fractions were then assayed for glycogen phosphorylase activity in the presence

and absence of 5′ AMP as described below. Column fractions were also assayed for protein kinase activities as explained below.

The overlapping phosphorylase *b* and enodgenous kinase fractions were concentrated and washed using a YM-30 (Amicon) membrane at 40 psi. The concentrate was applied to a Sephacryl 300 (S-300, Pharmacia) gel filtration column (90 cm x 2 cm) equilibrated in Buffer A containing 50 mM KCl and 5 mM EGTA at pH 8.5. Protein was eluted in the same buffer at 30 ml/hr and 2 ml fractions were collected. Column fractions were assayed for phophorylase *b* and protein kinase activity as described below.

3.2.2.4 Glycogen Phosphorylase Assay

A 20 μl enzyme sample was added to 200 μl of reaction mixture containing 50 mM imidazole (pH 6.8), 2.5 mg/ml glycogen, 5 mM $MgCl_2$, 0.5 mM NADP, 50 μg/ml glucose-1-6-diphosphate, 2 mM K_2HPO_4, 0.3 units/ml glucose-6-phosphate dehydrogenase, and 0.4 units/ml phosphoglucomutase. The temperature of the assay mixture was maintained at 23°C. A molar extinction coefficient of 6.2 x 10^3 was used to quantitate NADPH formation at 340 nm. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol NADPH/min at 23°C.

3.2.2.5 Protein Kinase Assay

A 40 μl sample of desalted concentrate obtained from the DE-52 column was mixed with an equal volume of reaction mixture containing 25 nM γ^{32}P-ATP (30 Ci/mmol) in Buffer A at pH 8.2 containing either casein at 2.5 mg/ml or no exogenously added substrate. The cations Mn^{2+} and Mg^{2+} were added at the desired final concentrations of 50 μm-10 mM. The total reaction volume was kept constant at 100 μl. All additions were carried out in an ice bath at 0°C. The entire mixture was incubated at 23°C for 5-7 min. At the end of the incubation period 50 μl aliquots were

removed to 1 cm squares of Whatman 3 MM paper. The filters were carried through a series of trichloroacetic acid (TCA) washes; wash 1, 10% TCA for 15 min at 4°C wash 2, 5% boiling TCA for 5 min, wash 3, 5% TCA at 23°C for 10 min. The filters were then washed in acetone, air dried and counted by liquid scintillation (Tm Analytic).

3.2.2.6 Antibody Preparation

Both the *a* and *b* forms of the phosphorylase were purified to homogeneity as determined by SDS-PAGE (Cloutier and Rutherford 1987). These proteins were subjected to preparative SDS-PAGE, and were then eluted from the gel slices electrophoretically. The eluates were used as antigens. Two New Zealand white rabbits were each injected with 50-150 μg of purified protein in complete Freunds adjuvant. The rabbits were reinjected with 50-100 μg protein in incomplete Freunds adjuvant at two week intervals. After 6 weeks, the presence of antibody to the protein was confirmed by western blotting. Whole blood was allowed to clot overnight at 4°C, then centrifuged at 10,000 x g for 10 min. The serum was divided into 300 μl aliquots and stored frozen at -20°C for subsequent use. Both antiserum preparations had similar titers; a 1:500 dilution of each serum was used for immunostaining and immunoprecipitation.

3.2.2.7 Western Blotting

Protein samples were prepared for SDS-PAGE by boiling in the presence of 2% SDS, 5% dithiothreitol (DTT) and 0.02% Pyronin Y. The samples were separated on 6% SDS-PAGE gels according to Laemmli (1973). Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes using a Hoefer transfer chamber and a power source (Model TE51) set at 1.2 amps for one hour. The transfer buffer contained 192 mM glycine and 20% methanol in 25 mM Tris-HCl, pH 8.3. After transfer, the nitrocellulose was placed in 50 ml of 50 mM Tris-HCL, pH 7.6, 200 mM NaCl and 0.1% Tween 20 (buffer D), and gently shaken for 20-30

min (Immunostaining method was a personal communication from Dr. Asim Esen, Biology Dept., Virginia Tech, Blacksburg, VA). The buffer was then replaced with antiserum diluted 1/500-1/170 in fresh buffer D. After 1-12 hr of incubation with the antibody, the nitrocellulose was rinsed several times with fresh 50 ml solutions of buffer D. A 50 ml solution of buffer D containing 1 μg/ml protein A-peroxidase was then placed onto the blot and incubated with gentle shaking for 1 hr. The nitrocellulose was rinsed with two 50 ml volumes of buffer D and then with 50 ml of buffer D lacking Tween 20 (buffer D′). The nitrocellulose was then exposed to 48 ml of peroxidase reaction mixture containing 17% methanol, 0.5 mg/ml of 4-chloro-1-napthol, and 0.008% H_2O_2 in buffer D′ for 30-120 min. In some cases, the blots were also stained for protein using 0.1% napthol-blue black (amido black) in methanol, water, and acetic acid (4.5:4.5:1 ratio, v/v/v). The destaining solution contained 90% methanol and 2% acetic acid. Autoradiography of the blots was performed using a Kodak intensifying screen and Kodak XAR-5 X-ray film.

3.3 Results

3.3.1 Factors Affecting Enzyme Activity and the Apparent Molecular Weight of Phosphorylase b as Determined by SDS-PAGE

We have previously shown that the early stages of *Dictyostelium* differentiation contain an inactive form of phosphorylase which requires 5'AMP for activity (phosphorylase *b*). In addition, we observed a 5'AMP independent phosphorylase *a* activity appearing in mid-late development (Rutherford & Cloutier 1986). The sum of the specific activities of the *a* and *b* forms of this enzyme remained constant at 0.02 μmols/min/mg protein. This value is similar to the maximum specific activity of *Dictyostelium* phosphorylase reported in the literature (Firtel & Bonner 1972; Gustafson & Wright 1972; Jones & Wright 1970; Marshall et al. 1970; Rutherford & Harris 1976; Wright et al. 1968; Higgins & Dahmus 1982).

Early in our studies we observed a differential stability of the phosphorylase *b* activity during the growth phase. For example, phosphorylase *b* activity from cells in the early logarithmic phase (2 x 10^6 cells/ml) retained only 15%-30% of its activity after storage at 4°C or -20°C for one day. The inclusion of the phosphatase inhibitor NaF (25 mM), and the protease inhibitor PMSF (2 mM) in these extracts did not improve the stability of the *b* enzyme. Phosphorylase *b* activity obtained from amoebae in the late logarithmic or early stationary phase was more stable to cold storage. Specifically, 80%-90% of the *b* enzyme activity was recovered after storage at 4°C or -20°C for one day. Therefore, all studies requiring *b* enzyme activity from amoebae utilized cells in the late logarithmic growth phase.

It was also observed that under different extraction conditions, the proteins detected with anti-b antiserum shifted from a singlet to a doublet as analyzed by SDS-PAGE. The singlet/doublet could be immunoprecipitated by the anti-b antiserum. The immunoprecipitation of the singlet/doublet resulted in inhibition of up to 90% of the phophorylase *b* activity (Naranan

et al. 1988). The singlet/doublet corresponded to the molecular weight of phophorylase *b* purified from amoeba as determined by SDS-PAGE. The detectability of the enzyme activity was determined by the extraction conditions. Previous studies on *Dictyostelium* phosphorylase utilized 5 mM phosphate buffer pH 6.8 and 50 mM phosphate buffer containing 10 mM 2-mercaptoethanol as extraction buffers, thus inactivating the enzyme (Jones & Wright 1970; Thomas & Wright 1976; Higgins & Dahmus 1982). This is undoubtedly one of the reasons why these reports failed to detect the *b* enzyme. We have already reported on other factors responsible for lack of detection of phosphorylase *b* activity by previous investigators (Rutherford & Cloutier 1986).

Since phosphoproteins show increased molecular weights upon SDS-PAGE with respect to their dephospho forms (Dahmus 1981), it was possible that the singlet versus doublet forms of the 92 kd *b* enzyme protein represented dephospho versus phospho forms of the phosphorylase *b*; i.e. the lower member of the 92 kd doublet protein may represent the dephosphorylated *b* form of the phosphorylase whereas the upper member of the doublet may represent the phosphorylated enzyme. This hypothesis was tested by preparing the slug stage extracts shown in Figure 11 in the presence or absence of various phosphatase inhibitors. Identical results were obtained in the presence or absence of these inhibitors (lanes 5-6 vs 7-10), suggesting that dephosphorylation was not responsible for conversion of the upper member of the 92 kd doublet into the lower member. Alternatively, it is possible that none of the phosphatase inhibitors tested, effectively inhibited a phosphorylase phosphatase. We have however, shown that 2 mM phenanthroline, 50 mM phosphate, and 5 mM para-nitrophenyl phosphate are potent inhibitors of *Dictyostelium* alkaline phosphatase as well as protein phosphatases that dephosphorylate phospho-kemptide (Ferris & Rutherford 1986). Kemptide is an artificial hexapeptide substrate for the assay of cAMP dependent protein kinases (Kemp et al. 1975). It is also possible that a particulate protease, or one that was pelletable at 100,000 x g, was responsible for degrading the 92 kd doublet form of phosphorylase *b* into the singlet form observed in 9,000 x g supernatants. We, therefore, tested the effect of including several different protease inhibitors in Buffer A (Buffer A was shown to be the best extraction buffer for the *b* enzyme). Inclusion of the following protease inhibitors during cell lysis had no effect on the molecular weight or activity of the *b* enzyme (PMSF, 170 μ g/ml; N-p-tosyl-l-lysine

chloromethyl ketone, 18μg/ml; benzamidine 300 μg/ml; N-tosyl-phenylalanine chloromethyl ketone, 17μg/ml; pepstatin A 1μg/ml) (data not shown).

3.3.2 Conversion of 5'AMP Dependent into 5'AMP Independent Activity in the Presence of Cations and ATP

Because we observed two forms of phophorylase activity in *Dictyostelium* corresponding to the mammalian enzyme and apparent shifts in molecular weight depending on buffer conditions, we decided to explore the possible regulation of phosphorylase through phosphorylation. This section describes the attempts made to demonstrate conversion of the 5'AMP dependent phosphorylase *b* into the 5'AMP independent *a* form by phosphorylation. To this end, the effects of exogenously added cations and ATP on the 5'AMP dependence of the *b* enzyme were monitored. Such an approach has been used to demonstrate phosphorylase kinases in other organisms (Fisher & Krebs 1955; Krebs & Fisher 1960). We initially attempted to demonstrate conversion of the *Dictyostelium* phosphorylase *b* in crude extracts or low and high speed supernatants. No conversion of the 5'AMP dependent form into a 5'AMP independent form was observed. Because the results from Figure 11 showed that the *b* enzyme activity was unstable in low speed supernatants, and because the method of preparation of cell free extracts affected the banding pattern of the phosphorylase *b* subunit, partially purified *b* enzyme obtained by DE-52 anion exchange chromatography was used for the following experiments. Fractions containing the phosphorylase *b* activity were pooled, concentrated and tested for conversion. Table 2 shows the effects of exogenously added nucleotides and cations on the the 5'AMP dependence of partially purified phosphorylase *b*. Preincubation of the sample with 4.7 mM ATP resulted in a four fold activation of total phosphorylase activity and a complete loss in the 5'AMP dependence of the *b* enzyme as measured spectrophotometrically (ATP effect). Equimolar amounts of GTP did not significantly affect the 5'AMP dependency, showing that the conversion was specific to ATP.

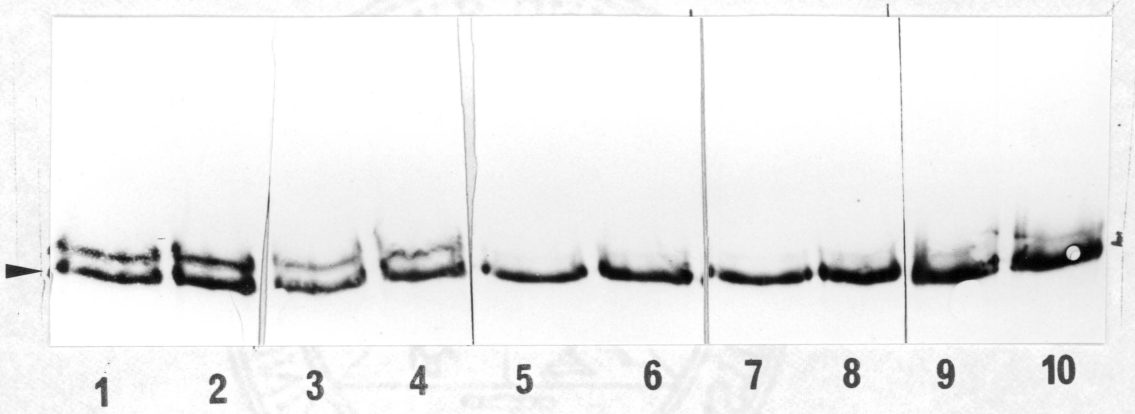

Figure 11. Effects of methods of sample preparation on the molecular weight of phosphorylase b: Cells at the migrating slug stage (0.1 grams wet weight) were lysed by freeze-thaw and harvested in buffer A in the presence or absence of phosphatase inhibitors. The lysates were centrifuged at 9,000 x g or 100,000 x g and the resulting supernatants assayed for phosphorylase activity as well as prepared for SDS-PAGE. The gel was then western blotted and probed with *Dictyostelium* anti-b antiserum. **Lanes 1,2**--100,000 x g supernatant, **Lanes 3,4**--100,000 x g supernatant recombined with 100,000 x g pellet that had been solubilized in 0.1% Triton X-100, **Lanes 5,6**--9,000 x g supernatant of cells lysed in the presence of buffer A, **Lanes 7,8**--9,000 x g supernatant of cells lysed in the presence of buffer A containing 50 mM NaF, **Lanes 9,10**--9,000 x g supernatant of cells lysed in the presence of buffer A containing 10 mM phenyl phosphate, 2 mM 1,10-phenanthroline and 60 mM potassium phosphate pH 7.0. The arrow indicates the position of the lower member of the 92 kd *b* subunit doublet protein.

Table 2. Effects o f Nucleotides and Cations on the 5'AMP Dependence of Phosphorylase "b"

	Treatment	Phosphorylase Activity[1] (nmol/min/ml) 5'AMP +	5'AMP -	Fold Dependence
A	Untreated "b" enzyme[2]	0.06	0.01	6
	"b" enzyme + 4.7 mM ATP[3]	0.23	0.28	nondependent
	"b" enzyme + 4.7 mM GTP[3]	0.17	0.04	4.2
B	Untreated "b" enzyme[2]	1.5	0.36	4.2
	"b" + 15 mM $MgCl_2$[4]	1.6	0.28	5.7
	"b" + 15 mM $CaCl_2$[4]	1.4	0.40	3.5
	"b" + 15 mM $MnCl_2$[4]	1.5	1.5	nondependent

[1] Each value represents an average of triplicates.
[2] Phosphorylase "b" activity from DE-52 chromatography of amoebal tissue was concentrated by ultrafiltration and desalted by washing in Buffer A.
[3] The "b" enzyme was preincubated with the appropriate nucleotide or cations for 30 min at 23 C and assayed for phophorylase activity in the presence/absence of 5'AMP.
[4] The "b" enzyme was preincubated with the appropriate cation for 5 min at 23 C prior to measurement of phophorylase activity.

We also tested the effects of exogenous cations on the 5'AMP dependence of phosphorylase *b*. In mammalian systems, addition of 1-10 mM Mg^{2+}, Ca^{2+}, or Mn^{2+} in the absence of exogenously added ATP was sufficient to activate phosphorylase kinase and result in conversion of phosphorylase *b* to *a*. This conversion occurred since micromolar concentrations of ATP endogenously present in these crude extracts were adequate for phosphorylase kinase activity (Fisher & Krebs 1955; Krebs & Fisher 1960). Table 2B shows that the addition of 15 mM Mn^{2+} in the absence of exogenously added ATP resulted in a total loss of 5' AMP dependence of the *Dictyostelium b* enzyme (addition of 5-7 mM Mn^{2+} also caused a similar effect). Addition of 1-10 μM ATP to these extracts did not enhance this apparent conversion of the *b* enzyme in the presence of Mn^{2+}. Thus, if the conversion were due to a Mn^{2+} stimulated phosphorylation, the endogenous ATP levels in the *Dictyostelium* extracts were sufficient to effect complete conversion. Furthermore, equimolar concentrations of Ca^{2+} and Mg^{2+} were without effect. The effect of Mn^{2+} on the 5'AMP dependence of the *b* enzyme was reversible by desalting the "converted" samples on a Sephadex PD-10 column. The reversibility of the Mn^{2+} effect could indicate that Mn^{2+} is an allosteric modulator of the *b* form rather than a cofactor for a kinase mediated phosphorylation. However, we found that purified samples of the *b* enzyme were not similarly affected by 7-15 mM Mn^{2+}, thus arguing against an allosteric modulation of the *b* enzyme by Mn^{2+}. Rather, purified samples of the *b* enzyme activity were completely inhibited under these conditions. Alternatively, the effect of Mn^{2+} on the 5'AMP dependence of the *b* enzyme may be due to phosphorylation by a protein kinase that itself has adequate levels of bound ATP to function.

3.3.3 Phosphorylation of Dictyostelium phosphorylase b in vitro and in vivo

Because the results in Table 2 suggested a cation-ATP mediated conversion of the *b* enzyme into a 5'AMP independent form, we examined these extracts for the presence of protein kinase activity. In this section, *in vitro* as well as *in vivo* phosphorylation of the 92 kd *b* enzyme subunit protein is demonstrated. Upon DE-52 chromatography of 100,000 x g supernatants from

the amoeba stage, we routinely observed a protein kinase activity that eluted from the column at a slightly lower salt concentration than that required to elute the phosphorylase *b* activity (the protein activity eluted at ≃ 150 mM KCl) (Figure 12). The protein kinase activity was initially assayed with casein as a substrate in the presence of 2.5 mM Mg^{2+} and 2.5 mM Mn^{2+}, prior to determining its preference for Mn^{2+}. The kinase showed a molecular weight of ≃ 200,000 daltons as determined by Sephacryl S-300 gel filtration chromatography (data not shown). The casein kinase activity was associated with an endogenous kinase activity (the activity observed when no exogenous substrate was included in the reaction mixture). The casein kinase appeared to phosphorylate a *Dictyostelium* substrate present in the DE-52 fractions. Both casein and endogenous kinase activities were undetectable in 100,000 x g supernatants of tissue from the amoeba stage, but were measurable following DE-52 chromatography. We had seen this inhibition of protein kinase activity previously in the purification of the cAMPdPK where activity only appeared after DE-52 chromatography (Rutherford et al. 1982). Figure 12 shows that the casein kinase activity peak (fractions 52-75) overlapped with a peak of endogenous kinase activity (frations 55-75). Both kinase activities overlapped with the *b* enzyme activity on the DE-52 column (The region of overlap is represented by fractions 60-80). It is noteworthy that the endogenous kinase activity peaked at the region of overlap between the casein kinase and phosphorylase *b* activities (fractions 65-70). Further, the ability of the kinase to phosphorylate casein was greatest when there was little or no overlap between casein, the artificial substrate, and phosphorylase *b*, the natural substrate, in the region of overlap (data not shown). The combined results of Table 2 and Figure 12 suggested that the *b* enzyme may serve as a substrate for the casein/endogenous kinase activities observed.

In Figure 13, we subjected the endogenous kinase and phosphorylase *b* activities to further purification with the aim of obtaining separate preparations of each enzyme. Figure 13 shows the results obtained when concentrated and washed DE-52 fractions containing casein kinase, endogenous kinase and phosphorylase *b* activities, were subjected to Sephacryl-S-300 gel filtration and tested for endogenous phosphorylation of the *b* enzyme. The S-300 column yielded adjacent peaks of endogenous kinase (fractions 45-60) and phosphorylase *b* activities (fractions 50-77). Thus, the peaks of the phosphorylase *b* and endogenous kinase activities were sufficiently separated, such

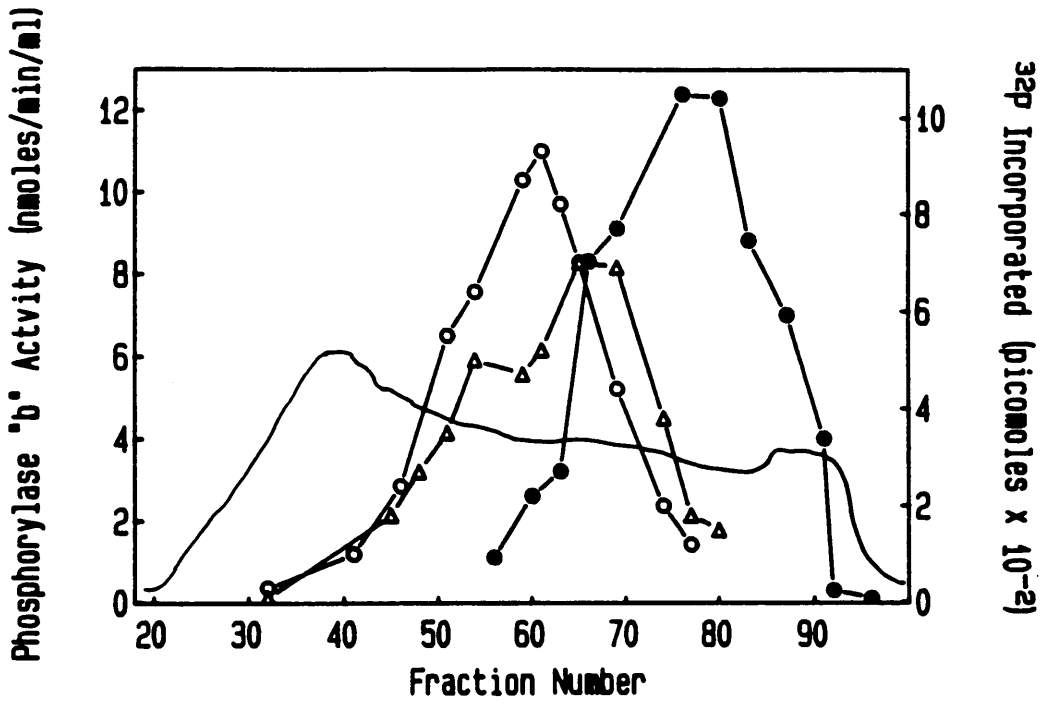

Figure 12. **Phosphorylase b and protein kinase activities from DE-52 chromatography of amoebal extracts:** The 100,000 x g supernatants of amoebae were subjected to chromatography on DE-52 cellulose as described in Methods. Column fractions were assayed for phosphorylase *b* (●), endogenous *b* kinase (△), and casein kinase (○) activities. The thin line represents protein concentration as measured at A_{280}.

that endogenous phosphorylation of the *b* subunit was detected only when the peak kinase (fraction 50) and peak phosphorylase *b* (fraction 65) activities were mixed (Figure 14A and B, Lane 5). The peak kinase activity (fraction 50) overlapped with the tail of the phosphorylase *b* peak, and therefore did not demonstrate endogenously phosphorylated 92 kd *b* enzyme subunit when tested without any additions (Figure 14A and B, Lane 11). In addition, the fraction containing maximal *b* enzyme activity (fraction 65) did not demonstrate endogenously phosphorylated *b* enzyme subunit when tested alone (i.e. without the addition of S-300 fractions containing kinase activity) (Figure 14A and B, Lane 13). Thus, the kinase and phosphorylase activities were separated sufficiently to demonstrate that the casein kinase did phosphorylate the 92 kd *b* enzyme subunit. Later in this report, we will present data showing that the phosphorylase *b* was the major substrate for the endogenous kinase in these fractions. The endogenous kinase will therefore be referred to as the *b* kinase for the remainder of this report.

In order to determine whether the endogenous phosphorylation of the *b* enzyme had physiological significance, or whether it was an *in vitro* phenomenon, we tried to demonstrate *in vivo* phosphorylation of the 92 kd *b* subunit. Amoebae were exposed to ^{32}P-orthophosphate for 1-2 hr, then lysed and subjected to a low speed centrifugation. We first attempted to immunoprecipitate the phosphorylated 92 kd *b* subunit from these low speed supernatants with *b* antiserum. The 92 kd *b* subunit could be immunoprecipitated with high recovery from crude extracts, as visualized by western blotting. However, only rarely was the incorporated ^{32}P retained with the protein during the lengthy immunoprecipitation protocol. We assumed that endogenous protein phosphatases removed the phosphate group from the peptide during immunoprecipitation. We, therefore, used a more rapid method that would enable detection of the phosphorylated 92 kd *b* subunit. Since 5'AMP Sepharose specifically binds the *b* enzyme via its 5'AMP binding site (Cloutier and Rutherford 1987), we mixed ^{32}P labelled cell extracts from radio-labelled amoebae with 5'AMP Sepharose. The phosphorylated 92 kd *b* subunit was found in the flow-through fraction, i.e. material that did not bind the 5'AMP resin (Figure 15B lanes 1-8). Bound phosphorylase *b* was eluted off the 5'AMP resin by pulsing it with 10 mM 5'AMP (Figure 15B lanes 9-16). The 92 kd *b* subunit eluted by 5'AMP was not phosphorylated and is therefore not shown on the

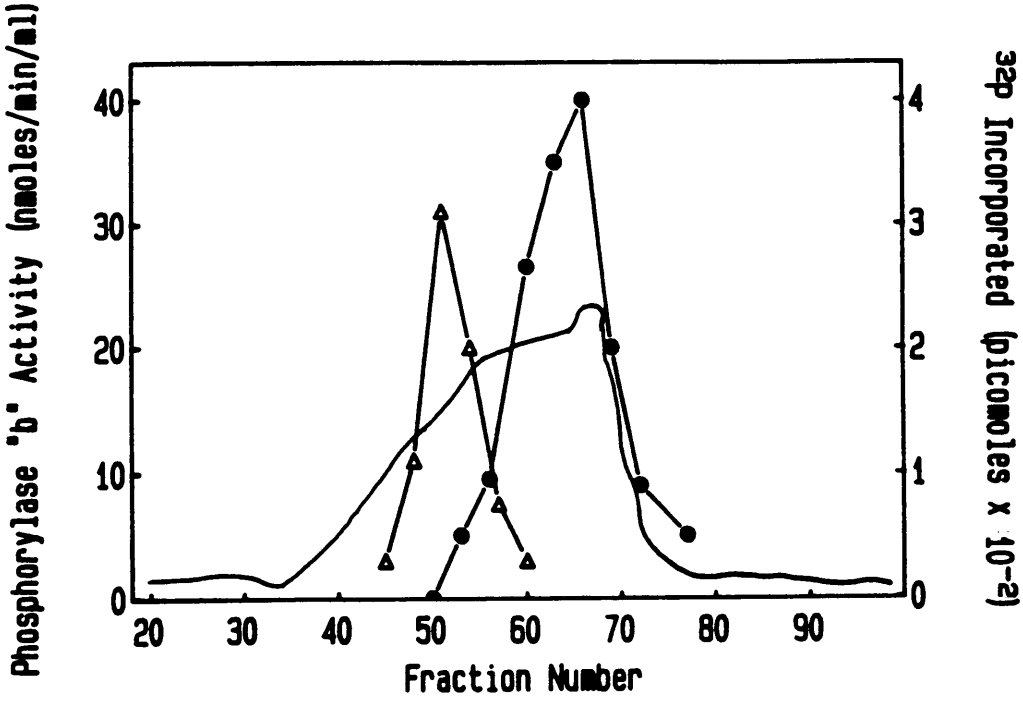

Figure 13. **Separation of Phosphorylase b and endogenous b kinase activities upon S-300 Gel Filtration:** A washed, DE-52 concentrate of the overlapping region containing phosphorylase *b* and endogenous *b* kinase was applied to the S-300 column. The column was eluted as described in Methods. Fractions were assayed for *b* kinase (△) and phosphorylase *b* (●) activities. The thin line represents protein concentration as measured by A_{280}. Fractions 45-57 contained *b* kinase activity whereas phosphorylase *b* activity was present in fractions 54-70. The peak *b* and *b* kinase activities were present in fractions 65 and 50 respectively.

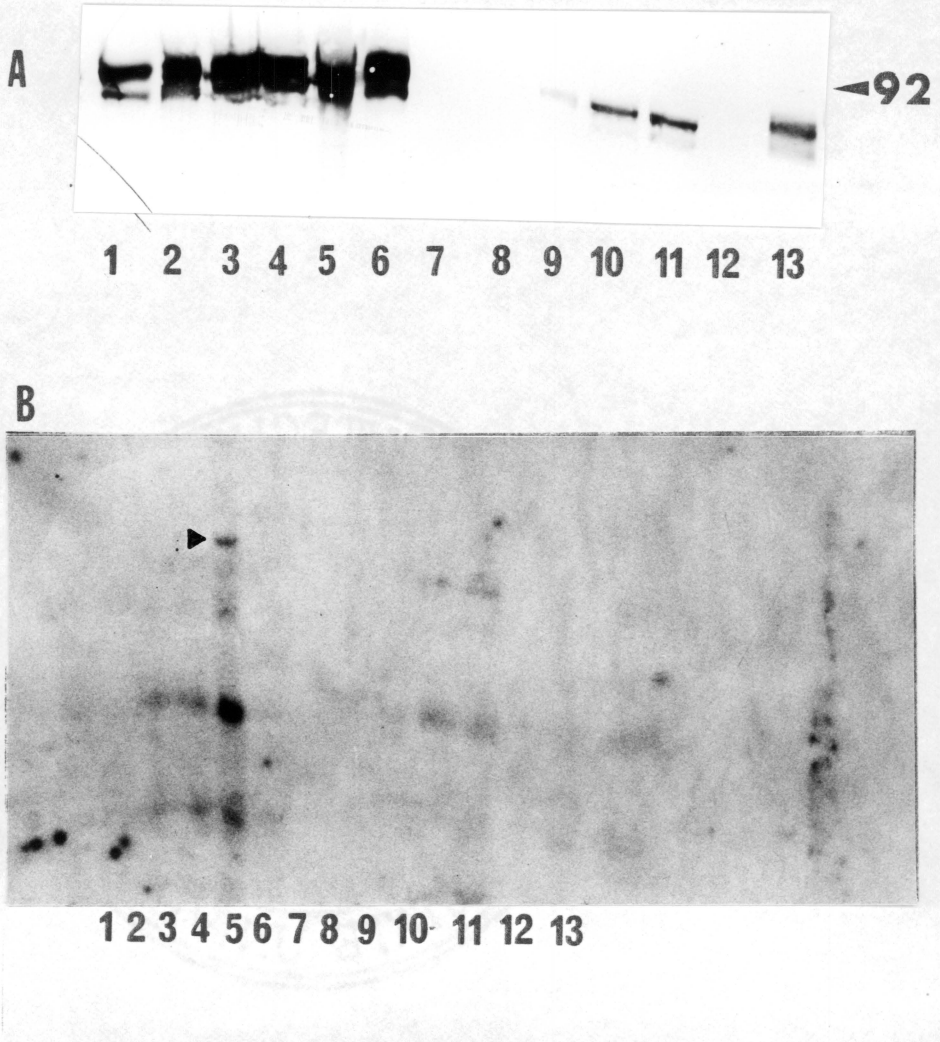

Figure 14. **In Vitro Phosphorylation of the 92 kd b subunit by the b kinase:** Phosphorylase *b* activity obtained by DE-52 chromatography of tissue from the amoeba stage was concentrated by ultrafiltration and desalted in buffer A. The concentrate was applied to a S-300 gel filtration column equilibrated in buffer A containing 50 mM KCl and 5 mM EGTA at pH 8.5. Column fractions were assayed for phosphorylase *b* and *b* kinase activities. Endogenous phosphorylation of the 92 kd *b* subunit was tested by mixing various fractions along the *b* kinase peak (fractions 45-57) with the fraction containing peak phosphorylase *b* activity (fraction 65) in the presence of 7 mM Mg^{2+}, 7 mM Mn^{2+} and 1.7 μm ATP (30 Ci/mol). The reactions were terminated by boiling in the presence of 2% SDS and 5% DTT and were then subjected to SDS-PAGE and western blotting. A Western blot of samples. **Lanes 1-3**– void volume S-300 fractions + fraction 65;, **Lanes 4,6**–tail of *b* kinase activity peak + fraction 65; **Lane 5**–peak *b* kinase fraction + fraction 65; **Lanes 7,8**–void volume S-300 fractions + buffer; **Lanes 9-11**–peak *b* kinase fractions + buffer; **Lane 12**–Blank; **Lane 13**–fraction 65 + buffer. **B** Autoradio the above western blot. Lanes are equivalent to those listed in panel A. The arrow indicates the position of the phosphorylated 92 kd *b* subunit protein.

autoradiogram in Figure 15B. The lack of detectable phosphorylated 92 kd *b* subunit obtained in 5'AMP eluate suggests that the phosphorylated *b* enzyme cannot bind to the 5'AMP resin. This inability to bind the 5'AMP resin may be due to a conformational change in the *b* enzyme as a result of phosphorylation. This explanation is supported by the observation that the late stage phosphorylase *a* enzyme does not bind to 5'AMP Sepharose under conditions in which the *b* form will (Cloutier & Rutherfore 1987). These data lend credence to the hypothesis that *Dictyostelium* phosphorylase *a* represents a phosphorylated form of the *b* enzyme. We found that the *b* enzyme was phosphorylated *in vivo* during the early developmental stages (1-15 hr into the developmental cycle). Optimal phosphorylation of the 92 kd *b* subunit was observed when cells at the amoeba or aggregation stage, (0.1 g wet weight) were pulsed with 1 mCi ^{32} P-orthophosphate for 0.5-1.5 hr (data not shown). Cells radiolabelled later in the differentiation cycle did not show a phosphorylated 92 kd *b* subunit protein. Indeed, it is significant that the observed *in vitro* and *in vivo* phosphorylation of the *b* enzyme occured in the amoeba stage of development prior to the appearance of *a* enzyme activity. (The *in vivo* phosphorylation top of the *b* form was also detected in the aggregation stage of development, just prior to detection of phosphorylase *a* activity: data not shown). These data were compatible with the hypothesis which states that phosphorylation is the mechanism of conversion of phosphorylase *b* into *a*. Taken together, the data in Figures 12-15 and Table 2 pointed to the existence of a *Dictyostelium* phosphorylase kinase which phosphorylates and converts the the phosphorylase *b* form into a 5'AMP independent form during early development.

3.3.4 Characterization of the casein and Endogoenous b kinase activities

In order to test the hypothesis that the endogenous *b* kinase and the casein kinase were the same enzyme, we characterized the biochemical requirements of the casein activity vs the endogenous *b* kinase activity. The kinase activities were obtained from DE-52 chromatography of amoeba stage cells. Since mammalian phosphorylase kinases characteristically show an activation

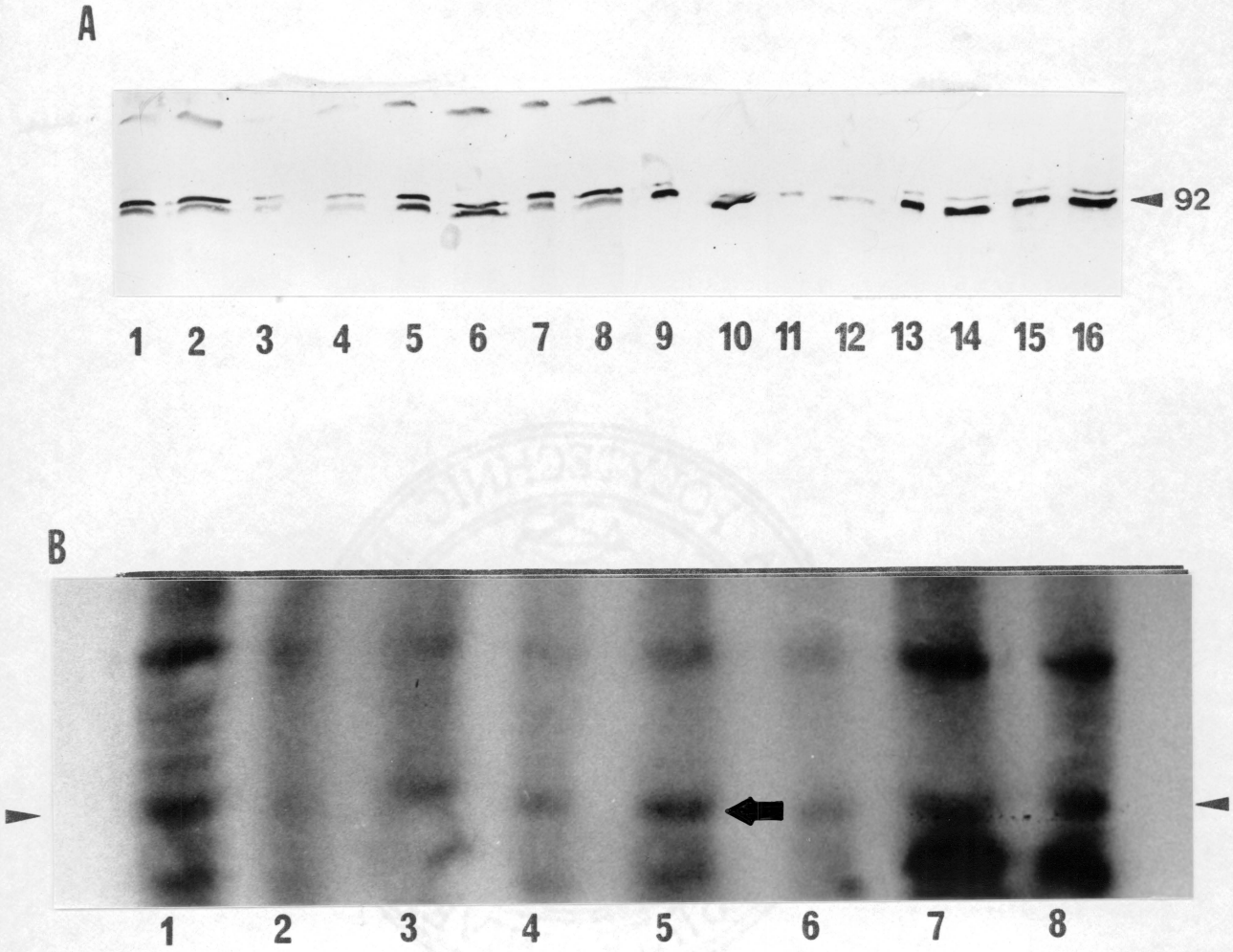

Figure 15. Phosphorylation of the 92 kd b subunit in vivo: Logarithmically growing amoebae (0.1 grams wet weight) were plated onto Gelman filters in buffer B. The amoebae were allowed to develop for 1 or 3 hr prior to being radiolabelled. Each filter of cells was labelled with 1 mCi ^{32}P-orthophosphate for 0.5 or 1.5 hr. The cells were lysed by freeze-thaw in 1 ml of buffer A containing 5 mM EGTA. Each lysate was centrifuged at 10,000 x g for 5 min. and mixed with 0.4 ml of 5′AMP Sepharose for 20 min at 4°C. This mixture was centrifuged at 10,000 x g for 5 min. The bound proteins were eluted off the resin with a pulse of 10 mM 5′AMP. Both the 5′AMP flow-through and eluate samples were prepared for SDS-PAGE and western blotting. The resulting immunoblot (A) and its autoradiogram (B) are shown **Lanes 1-2**--5′AMP eluate of 1 hr cells labelled for 0.5 hr; **Lanes 3-4**--5′AMP eluate of 1 hr cells labelled for 1.5 hrs; **Lanes 5-6**--5′AMP eluate of 3 hr cells labelled for 0.5 hr; **Lanes 7-8**--5′AMP eluate of 3 hr cells labelled for 1.5 hrs; **Lanes 9-10**--5′AMP flow through of 1 hr cells labelled for 0.5 hr; **Lanes 11-12**--5′AMP flow through of 1 hr cells labelled for 1.5 hr; **Lanes 13-14**--5′AMP flow through of 3 hr cells labelled for 0.5 hr; **Lanes 15-16**--5′AMP flow through of 3 hr cells labelled for 1.5 hr; The arrows indicate the position of the phosphorylated phosphorylase 92 kd *b* subunit.

at pH 8.2 versus 6.8, we measured the casein and endogenous kinase activities at pH 6.8 and 8.2 in the presence of various concentrations of Mn^{2+}. Both casein and endogenous protein kinases showed low activity when assayed with similar concentrations of Mg^{2+}. Figure 16 shows that both the casein and *b* kinase activities show an activation at pH 8.2 in the presence of 1-5 mM Mn^{2+}. Figure 16 also shows that the casein kinase activity was activated when assayed in the presence of 1-5 mM Mn^{2+} at pH 8.2. as compared with its activity when assayed at pH 6.8. Likewise, the endogenous *b* kinase was more active when assayed in the presence of 1-5 mM Mn^{2+} at pH 8.2 than at 6.8 (Figure 16). Thus, Figure 16 shows that both kinases show maximum activity when assayed with Mn^{2+} at pH 8.2.

Phosphorylase *b* was optimally phosphorylated under conditions where the *b* kinase and casein kinase were active, that is, in the presence of 1-5 mM Mn^{2+} at pH 8.2. Phosphorylation of the *b* enzyme was undetectable in the presence of identical concentrations of Mg^{2+}. Although low kinase activity was measured at pH 6.8 (Figure 16), no endogenous phosphorylation of the 92 kd *b* enzyme subunit was detected at this pH (data not shown). Thus, the similar pH and cation requirements of the two kinases lend further support to the hypothesis that the *Dictyostelium* casein kinase also phosphorylates phosphorylase *b*. The only protein that is phosphorylated in the region of overlap between casein kinase and phosphorylase *b* activities is the phosphorylase *b* 92 kd subunit (Figure 12, fractions 65-70). Therefore, as discussed in the previous section, the endogenous kinase activity observed in figures 14 and 16 is entirely a result of ^{32}-P incorporation into the phosphorylase *b* protein.

In addition, the substrate specificities of the casein and *b* kinase activities were also investigated. Both activities were associated with histone kinase activity; in addition, the *b* kinase activity was capable of phosphorylating protamine sulfate (data not shown). Both casein and *b* kinase activities were unaffected by 10μM cAMP and 10μM cGMP. In addition, Ca^{2+} and calmodulin (3 mM and 20 units respectively) failed to activate both kinase activities (data not shown). In order to provide additional evidence that the two kinase activities represent a single enzyme, we added phosphorylase *b* purified to homogeneity, back to partially purified casein kinase preparations. No phosphorylation of the added *b* form was observed as measured by autoradiography. The lack of

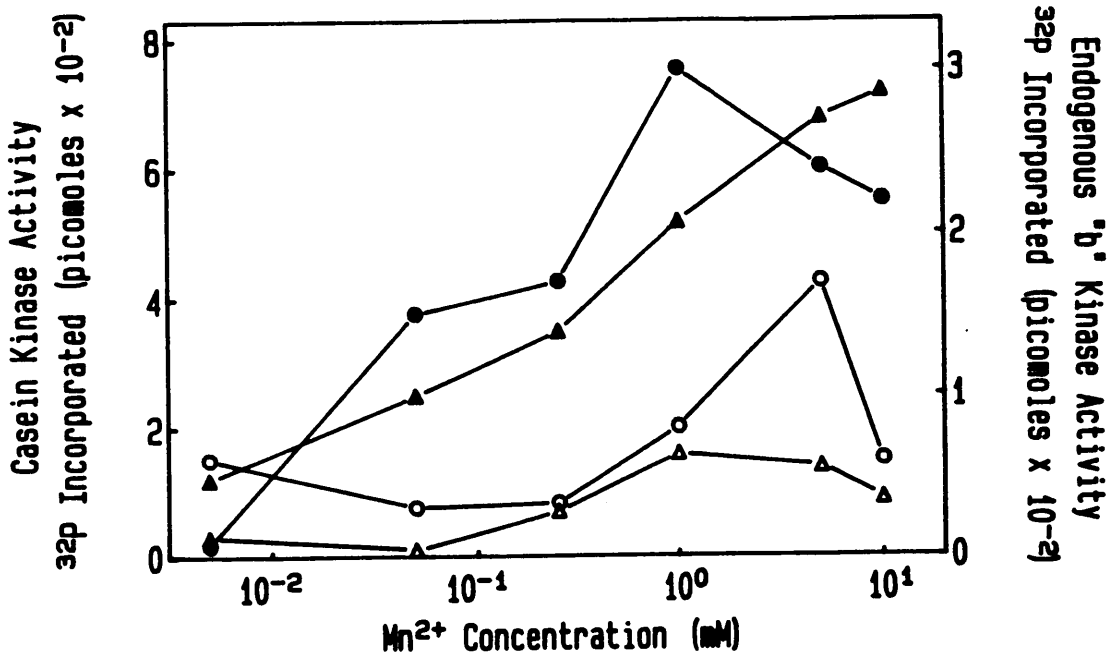

Figure 16. pH and Cation Requirements for Casein Kinase and Endogenous b Kinase Activities: Casein kinase activity or endogenous *b* kinase activity purified through DE-52 cellulose chromatography was concentrated and desalted into buffer A at pH 8.2 or buffer C at pH 6.8. The desalted enzyme sample was assayed for casein kinase activity (△,▲) or endogenous *b* kinase (○, ●) activity in the presence of increasing concentrations of Mn^{2+} at pH 6.8 (open symbols) or pH 8.2 (closed symbols).

phosphorylation of the *b* enzyme in this case may be due to alteration of the *b* form during purification. Alternatively, loss of an essential cofactor during purification of the *b* enzyme may result in inability of the endogenous *b* kinase to phosphorylate its substrate. We have also attempted conversion of the *Dictyostelium b* enzyme by rabbit muscle phosphorylase kinase. As a result of several experiments, we conclude that the *Dictyostelium b* enzyme is not a substrate for rabbit muscle phosphorylase kinase under conditions in which rabbit phosphorylase *b* shows conversion to the *a* form.

3.4 Discussion

The data presented, show that the *Dictyostelium* phosphorylase *b* from amoeba can be phosphorylated *in vivo* and *in vitro*. The *in vitro* phosphorylation of the *b* enzyme occurred under conditions which resulted in the apparent conversion of phosphorylase *b* activity into *a* activity (Table 2). Specifically, loss of 5'AMP dependence of the *b* form and detection of the phosphorylated 92 kd *b* subunit protein *in vitro* occurred only in the presence of 1-10 mM Mn^{2+} (Table 2).

The endogenous *b* protein kinase appeared similar to a *Dictyostelium* casein kinase with respect to its pH and cation requirements. The casein kinase required pH 8.2 and 1-5 mM Mn^{2+} in order to phosphorylate casein. Phosphorylation of the *b* enzyme however, also occurred in the presence of 1-5 mM Mn^{2+} at pH 8.2. It is interesting to note at this point, that several mammalian casein kinases are capable of phosphorylating the mammalian *b* enzyme (Delange et al. 1968). The *Dictyostelium* casein kinase reported by Renart is a Mg^{2+} dependent nuclear enzyme, and is therefore presumably not equivalent to the cytosolic, Mn^{2+} dependent kinase activity reported here (Renart et al. 1984). The phosphorylation of the *b* enzyme was also detected *in vivo*, suggesting that the observed *in vitro* phosphorylation of this enzyme was physiologically significant. Since the late stage phosphorylase activity is first detectable in the late aggregation stage of development, it is perhaps significant that the *in vivo* phosphorylation of the *b* enzyme occurred at the amoeba and aggregation stages. Interconversion between the two forms of phosphorylase by phosphorylation-dephosphorylation would explain our previous data: for example, we have consistently observed that the sum of the specific activities of the *b* and *a* forms remains constant during *Dictyostelium* development (Rutherford & Cloutier 1986). Secondly, we have observed cAMP induction of a 5'AMP independent phosphorylase *a* activity, suggesting that a phosphorylation cascade initiated by the cAMP dependent protein kinase may be regulating the interconversion between the two forms of *Dictyostelium* phosphorylase (Brickey et al. Submitted).

On the other hand, our previous work has shown that the partially purified late stage phosphorylase has a subunit molecular weight of 104 kd. Since phosphorylation of the 92 kd *b* enzyme subunit did not appear to alter its molecular weight, we cannot conclude that the apparent activation of the *b* enzyme resulted in production of the late stage phosphorylase (Table 2). However, phosphorylation of rabbit muscle phosphorylase *b* results in activation of the enzyme without a concommitant shift in its molecular weight. It is clear, however, that a 5'AMP independent activity, and a phosphorylated 92 kd *b* subunit are detected only in the presence of 5-15 mM Mn^{2+} (Table 2). Therefore, it is possible that incomplete phosphorylation of the *b* subunit was obtained. Indeed, it is entirely possible that the two forms of *Dictyostelium* phosphorylase represent separate gene products, one of which can be additionally modulated by phosphorylation. This mechanism of regulation would allow for a coarse and fine control of phosphorylase activity and therefore glycogen levels during development. In particular, the *in vivo* phosphorylation of phosphorylase *b* from the amoeba stage may represent an example of fine control, i.e. phosphorylation of the *b* form could activate the enzyme during early development, when the active *a* enzyme is absent. This mechanism of fine control would enable glycogen degradation during early development if it were required, perhaps as an energy source for migrating amoebae. According to this model, the late stage phosphorylase would be regulated at the level of protein synthesis, thus reflecting the use of glycogen as a source of glucose for the synthesis of the cellulose during differentiation. This dual mechanism may be effective, perhaps essential, for an organism which uses glycogen for the synthesis of developmentally regulated end products. We are currently investigating these ideas by sequencing the two forms of the phosphorylase and cloning their gene(s).

4.0 Partial Purification and Characterization of Glycogen Synthase I in Dictyostelium discoideum

Abstract

The partial purification and characterization of glycogen synthase *I* and its relationship to glycogen synthase *D* represents a preliminary study to determine whether its regulation may be through a cAMP-dependent phosphorylation event as occurs in other eucaryotes. Glycogen synthase *I* was partially purified through step NH_4SO_4 precipitation, and DE-52 anion exchange and Sephacryl 300 gel filtration chromatographies. The enzyme activity became increasingly unstable with purification, thus preventing purification to homogeneity. The partially purified glycogen synthase *I* activity was characterized as to the effects of pH, salts, phosphate, and substrate concentrations. Glycogen synthase *I* had a pH optimum between 7.5 and 8.0, slightly lower than rabbit muscle enzyme's pH optimum of pH 8.0-8.5. The substrate Km for glycogen and UDPG were similar to the rabbit muscle glycogen synthase at 2.8 mM for UDPG and 0.8 mg/ml for glycogen. Inhibition by the salts NaCl and KCl reached 50% inhibition at 100 mM and 175 mM respectively. Previous researchers had observed phosphate inhibition in 122,000 x g supernatants for glycogen synthase

D activity. However, we found this phosphate inhibition to disappear upon further purification of glycogen synthase *I*. Glycogen synthase *I* was found primarily in the cytoplasm and was the predominant form throughout the developmental cycle. However, glycogen synthase *D* activity was bound to the glycogen pellet and never found in the cytoplasmic fraction. In disagreement with previous reports, glycogen synthase *D* activity peaked early in development and decreased rapidly after cell aggregation. Glycogen synthase *I*, in either 100,000 x g supernatants or partially purified column fractions, was unaffected by the addition of Mg^{2+}-ATP and/or partially purified cAMP-dependent protein kinase. Although, the expected conversion of glycogen synthase *I* to *D* did not occur under these conditions this does not rule out the regulation of glycogen synthase by a phosphorylation-dephosphorylation cycle.

4.1 Introduction

In *Dictyostelium*, glycogen plays a unique role in cellular differentiation. Glycogen doesn't serve as an energy source as it does in other organisms, but instead cellular proteins provide energy for differentiation (reviewed in Loomis 1975). In *Dictyostelium*, glycogen provides glucose units that are required for the synthesis of mucopolysaccharide in spores and for the synthesis of cellulose cell walls in both spore and stalk cells. Thus, the regulation of glycogen metabolism is involved in the differentiation of both cell types. Because of this unique role of the glycogen metabolic pathway, we became interested in the regulation of glycogen metabolism as a target for cAMP-dependent protein kinase (cAMPdPK). Specifically, I wished to determine if the two *Dictyostelium* glycogen synthase activities, *I* and *D*, could be regulated via a phosphorylation-dephosphorylation cycle in response to cAMP levels.

Wright and Dahlberg (1967) and later Hames et al. (1972) described two glycogen synthase actvities in *Dictyostelium*. The two forms were termed *I* and *D* forms due to their similarity to the two forms of glycogen synthase previously characterized in mammals. The *D* form (Dependent form) required glucose-6-phosphate (G-6-P) for activity. The *I* form (Independent form) was active in the presence or absence of G-6-P. In mammals, these two forms of glycogen synthase were regulated by cAMP via a cAMPdPK. Therefore, it seemed likely that the *Dictyostelium* enzyme was regulated in a similar manner.

Saunders and Wright (1977) partially purified and characterized the *D* form of *Dictyostelium* glycogen synthase. However, a consideration of the relationship of the two forms of synthase activity was not discussed. At the time of Saunders and Wright's (1977) investigation of glycogen synthase *D*, a *Dictyostelium* cAMPdPK had not been found and there was considerable doubt of its existence. It was not until 1982 that several labs described a *Dictyostelium* cAMPdPK (Rutherford et al. 1982; Leichtling et al. 1982; de Gunzburg & Veron 1982; Schoen et al. 1983). This discovery renewed interest in the regulation of enzyme and gene activity in *Dictyostelium* by cAMP via this cAMPdPK. I report here on the further characterization of a partially purified form

of glycogen synthase *I*, its characteristics in comparison to mammalian glycogen synthase, and its relationship to the G-6-P dependent form during development.

4.2 Materials and Methods

4.2.1 Materials

^{14}C-labelled uridine diphosphoglucose (UDPG) was obtained from New England Nuclear (Dupont), Wilmington, DE. DE-52 anion exchange resin and 3MM paper were purchased from Whatman, Inc. Clifton, NJ. Sephacryl 300 resin and disposable desalting columns (PD-10) were purchased from Pharmacia LKB Biotechnology Inc., Piscataway, NJ. Acrylamide and molecular weight standards were from Bio-Rad Laboratories, Rockville Centre, NY. YM-10 membranes were from Amicon Corp., Lexington, MA. Other chemicals were from Sigma Chemical Co., St. Louis, MO.

4.2.2 Methods

4.2.2.1 Harvesting of Cells and Preparation of Extracts

Dictyostelium discoideum (AX-3) was grown in liquid HL5 media on a rotary shaker as previously described (Sussman and Sussman 1967). The resulting amoebae were harvested by centrifugation in a continuous flow rotor, then washed and resuspended in 50mM Tris-HCl, pH 7.5, containing 2 mM benzamidine, 2 mM mercaptoethanol and 0.02% sodium azide (Buffer A). Washed cells were resuspended in 3 volumes of cold buffer A and sonicated by subjection to four, one minute exposures from a 2 cm probe of a sonic cell disruptor (Model 300, Fisher). This lysate was centrifuged for 45 min at 100,000 x g. The resulting supernatant was treated with neutralized protamine sulfate at a concentration of 3 mg/g wet weight of cells. After stirring for 5 min, the

supernatant was centrifuged for 45 min at 100,000 x g to remove nucleic acids. The supernatant was then carried through further purification as described below.

4.2.2.2 Partial Purification of Glycogen Synthase I

The protamine sulfate treated 100,000 x g supernatant was NH_4SO_4 precipitated by slowly adding solid NH_4SO_4 to a 10% final concentration. After stirring on ice for 30 min, the supernatant was centrifuged at 10,000 x g for 30 min in a Sorvall RC-5B centrifuge. The pellet was resuspended in Buffer A and dialyzed overnight. The above procedure was repeated twice on the supernatant but with a 25% and a 35% final NH_4SO_4 concentration. The 25% and 35% pellets were resuspended in 150 mls of Buffer A (sufficient to dilute the NH_4SO_4 to a non-interfering level) and batch treated with 0.2 volumes of DE-52 resin that had been equilibrated in buffer A. A portion of the remaining supernatant was retained and dialyzed overnight for enzyme assay. The flow-through fractions of these two columns (designated A for 25% and B for 35%) were then brought to a 70% NH_4SO_4 final concentration. The pellets were resuspended in 3 ml of Buffer A containing 50mM KCl and loaded onto separate S-300 columns (1.6 x 90 cm, flow rate 30 ml/hr, 2 ml fractions). The active fractions from each column were pooled and concentrated with an Amicon YM-10 membrane. Fraction A was used for further characterization of the enzyme.

4.2.2.3 Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on samples from each of the purification steps as described by Laemmli & Favre (1973) using 6% resolving gels and 3 % stacking gels. The gels were silver-stained for protein according to the method of Merril et al. (1981). Nondenaturing tube gels were stained for enzyme activity according to the method of Krisman and Blumenfeld (1986).

2.2.2.4 Glycogen Synthase Assay

A 25 μl sample of the enzyme extract was mixed with an equal volume of reaction mixture containing, 50 mM Tris-HCl pH 8.2, 5 mg/ml glycogen, 3 mM UDPG, ^{14}C-UDPG (Specific Activity-210 mCi/mmole), either in the presence or absence of 5 mM glucose-6-phosphate (G-6-P). Incubation was carried out in a room temperature water bath for 30 min. The entire sample was spotted onto 1 cm squares of 3MM Whatman paper and immediately placed in ice cold 66% ethanol for 30 min followed by a second ice cold 66% ethanol wash for 30 min and finally a 5 min acetone bath (Thomas et al. 1968). The filters were then air-dried, placed in Ecoscint scintillation fluid, and the radioactivity determined on a TM-analytic scintillation counter. The differences in the activity of glycogen synthase *I* vs glycogen synthase *D* were based upon the requirement of glycogen synthase *D* for G-6-P. Additions made during the characterization of glycogen synthase *I* were such that the concentrations of the components of the reaction mixture were unaffected.

4.2.2.5 Measurement of Enzyme Activity During Growth and Development

Cells were harvested at intervals during the growth phase and assayed for enzyme activity. A 30 ml sample was harvested by centrifugation, then washed and resuspended in Buffer A (1:3 weight:volume). The cells were broken by three 45 sec treatments with a sonic cell disruptor (Model 300, Fisher), then centrifuged at 8,000 x g for 20 min. The resulting supernatant was centrifuged for 1 hr at 100,000 x g to pellet the glycogen. The glycogen pellet was resuspended in a volume equal to the supernatant and both fractions were assayed for enzyme activity.

When differentiated cells were required, the amoebae were washed free of media, pelleted by centrifugation and diluted 1:3 (weight:volume) in 7 mM N-morpholinoethanesulfonic acid (pH 6.5) containing 20 mM KCl and 5 mM $MgSO_4$ (Buffer B). Then 0.4 ml of this suspension was plated onto Gelman GN-6 membrane filters supported by Gelman absorbent pads. Both membranes and pads were pre-soaked for 30 min in Buffer B. At the required stage of development, the

membranes and supported cells were frozen at -70°C. Upon thawing, 0.5 ml of Buffer A was applied to each pad, the lysates were scraped from the membranes, and then centrifuged at 8000 x g for 15 min. The 8000 x g supernatant was removed, then centrifuged again at 100,000 x g for 30 min. The glycogen pellet was resuspended in Buffer A to a volume equal to the supernatant and both were assayed for glycogen synthase activity.

4.3 Results

4.3.1 Partial Purification of Glycogen Synthase I from Amoebae

Glycogen synthase *I* was partially purified by the method outlined in Figure 17. Buffer A contained 10% sucrose in order to prevent breakage of lysozomes during sonication. The protamine sulfate precipitation of nucleic acids resulted in significant clearing of nucleic acids and other charged particles from the 100,000 x g supernatant which could interfere in ion exchange chromatography. Glycogen synthase activity was unaffected by protamine sulfate precipitation. The *I* form of glycogen synthase did not adsorb to the glycogen pellet as occurs in other systems but was always found in the cytoplasmic fraction. Although glycogen synthase *I* did not bind to the glycogen pellet, glycogen was still required as a primer for the enzymatic reaction.

Saunders and Wright (1977) had previously shown that 37.5% NH_4SO_4 would precipitate glycogen synthase from a 122,000 x g supernatant. This treatment resulted in the precipitation of a considerable amount of contaminating protein when used for the purification of the *I* form. We, therefore, used a series of step NH_4SO_4 precipitations--10%, 10%-25%, and finally 25%-35%. No glycogen synthase activity was precipitated at 10% NH_4SO_4, 40% of the activity precipitated in the 10%-25% fraction with the remaining 60% precipitating at the 35% step. No glycogen phosphorylase activity was precipitated at any of these steps. The two preparations, were suspended 10-25% (Fraction A) and 35% (Fraction B) NH_4SO_4 pellets (5 ml each), diluted in 150 ml of Buffer A, and loaded onto separate DE-52 anion exchange columns equilibrated in Buffer A. In both cases, glycogen synthase did not adsorb to the column (> 90% recovery in flow-through). If the pH of Buffer A was increased to pH 8.5, 15%-20% of the activity was adsorbed to the resin. Increasing the pH above 8.5 resulted in loss of the applied enzyme activity. The DE-52 supernatants (pH 7.5) were precipitated with 70% NH_4SO_4, the pellets were resuspended in Buffer A containing 50 mM KCL and subjected to chromatography on separate S-300 columns.

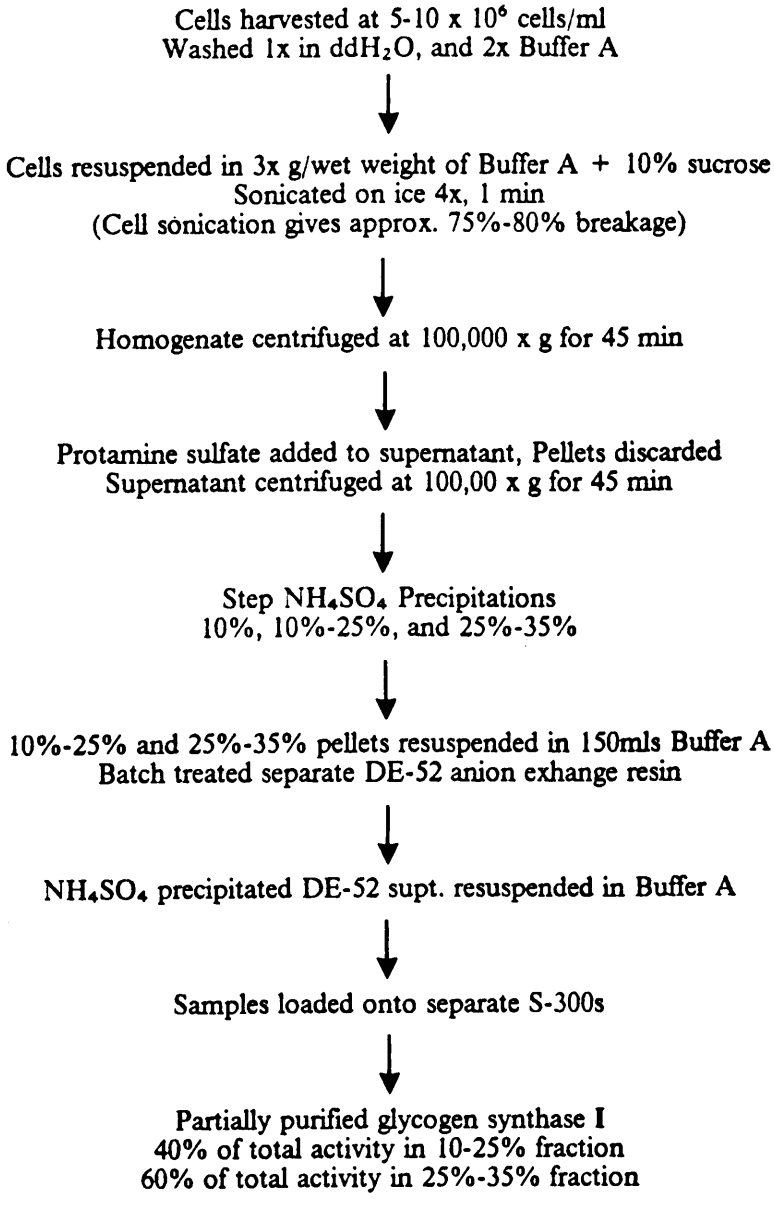

Figure 17. **Purification Scheme for Glycogen Synthase I:** Post S-300 purification such as hydroxylapatite and additinal anion/cation exchange chromatographies resulted in loss of activity. Affinity chromatography techniques used to purify rabbit glycogen synthase such as glycogen sepharose and glucosamine-6-phosphate affi-gel resins were ineffective in adsorbing the enzyme.

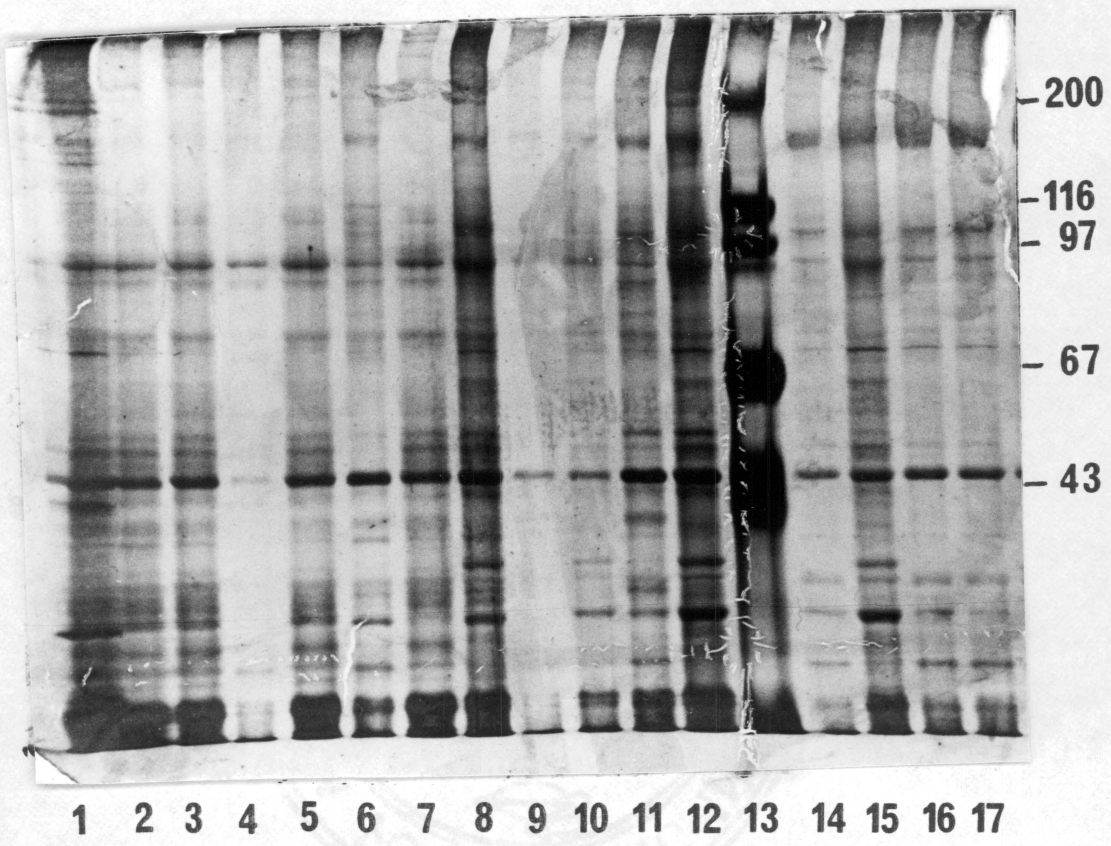

Figure 18. Silver-Stained SDS-PAGE of Purification Steps: Samples from each step of the purification scheme were diluted 1:40 in Buffer A except for samples from the two S-300 columns which were diluted 1:20 in Buffer A. These were then prepared for SDS-PAGE and 25 Ml of sample was loaded/lane. **Lane 1**-First 100k supt; **Lane 2**-Protamine sulfate treated 100k supt; **Lane 3**-10% NH_4SO_4 supt (post-dialysis, P.D.); **Lane4**-10% NH_4SO_4 pellet P.D.; **Lane 5**-10%-25% NH_4SO_4 supt P.D.; **Lane 6**-25% NH_4SO_4 pellet P.D.; **Lane 7**-25%-35% NH_4SO_4 supt P.D.; **Lane 8**-25%-35% NH_4SO_4 pellet P.D.; **Lane 9**-10%-25% NH_4SO_4 DE-52 Flow-through; **Lane 10**-25%-35% NH_4SO_4 DE-52 Flow-through; **Lane 11**-On S-300 of Lane 9; **Lane 12**-On S-300 of Lane 10; **Lane 13**-Molecular weight markers, sizes are indicated on the right; **Lane 14**-Pooled peak of Lane 11; **Lane 15**-Pooled peak of Lane 12; **Lanes-16 and 17** are peak fractions 41 and 43 of Lane 15, respectively.

The silver-stained SDS-PAGE of this partial purification is shown in Figure 18. We used the Fraction A S-300 pooled and concentrated peak for further characterization of the enzyme because it exhibited a sharp peak of activity rather than the broad peak of activity shown by the Fraction B when chromatographed on S-300. The Fraction A S-300 pooled peak was stable for several days when stored at 4°C. However, it was unstable to further purification. Saunders and Wright (1977) found that the addition of glycogen, UDPG, or G-6-P stabilized partially purified glycogen synthase *D*. We found that these additions did not increase the stability of the *I* form significantly. In mammals, glycogen synthase is often purified as a heterogeneous mixture of dimers, trimers, and tetramers having subunit molecular weights ranging from 87 kd-95 kd (Takeda et al. 1975). Although the pooled S-300 sample is obviously a heterogeneous mixture of proteins at this stage of purification (pooled fractions 38-45, Lane 14, Figure 18), there were two proteins in the 87-95 kd subunit molecular weight range of the mammalian glycogen synthase; an $\simeq$ 95 kd protein and an $\simeq$ 90 kd protein. Also present and coinciding with fractions containing enzyme activity was an $\simeq$ 180 kd protein that could be a dimer of the 90 kd band if insufficient denaturation of the native enzyme by the SDS had occurred. A sample of this concentrated S-300 peak was applied to non-denaturing 6% polyacrylamide tube gels which were subsequently stained for activity revealing a doublet. However, we was unable to elute sufficient enzyme from this gel slice to determine subunit molecular weight on SDS-PAGE.

Figure 19A shows the elution profile from S-300 chromatography of Fraction A. The elution volume corresponded to an approximate molecular weight of 280 kd. Fraction B glycogen synthase activity eluted in a broad peak from the same column (Figure 19B). The elution pattern of this broad peak of glycogen synthase activity may indicate heterogeneous combinations of enzyme subunits, thus making a molecular weight estimation impossible.

4.3.2 Characterization of the Partially Purified Glycogen Synthase I

Because S-300 chromatography of the Fraction A gave a well defined peak of activity, the peak fractions were pooled and concentrated for characterization of the enzyme. The *in vitro* pH optimum of the *Dictyostelium* enzyme (Figure 20) was similar to rabbit glycogen synthase with the greatest activity between pH 7.5 and 8.0 (Hizukuri and Larner 1964). This is also the same *in vitro* pH optimum range determined for the *D* form by Saunders and Wright (1977). The intracellular pH of *Dictyostelium discoideum* has been determined to be approximately pH 6.2 (Loomis 1977). This would tend to indicate a low level of glycogen synthase activity at the pH within the cell if the *in vitro* and *in vivo* pH optima were similar but does not exclude the possibility that glycogen synthase is compartmentalized in a cellular organelle in which the pH is maintained at or near the pH optimum of glycogen synthase.

Saunders and Wright (1977) showed that the *D* form of the enzyme was activated 26% by 200 mM KCl or NaCl. Therefore, we tested the *I* form activation by salt. However, Figure 21 shows that the *I* form of the enzyme was inhibited by NaCl and KCl, 50% inhibition occurred at 100 mM while at 500 mM the enzyme was nearly inactive. Enzyme activity was regained after dilution or desalting of the sample. Therefore, the lack of synthase activity in salt gradients of DE-52 anion exchange columns was not because of irreversible denaturation due to elution by KCl.

Saunders and Wright (1977) found that glycogen synthase *D* activity was completely inhibited by potassium phosphate in 122,000 x g supernatants. Figure 22 shows that inhibition of the *I* form of the enzyme by potassium phosphate is dependent upon the fraction that was assayed. In 100,000 x g supernatants phosphate inhibited at low levels (5mM) but with further purification phosphate inhibition decreased. The enzyme from 100,000 x g supernatants and from 10%-25% DE-52 flow-through fractions were 50% inhibited by $\simeq$ 50 mM. Further purification of the enzyme through S-300 chromatography yielded a sample that showed only 30% inhibition at greater than 300 mM.

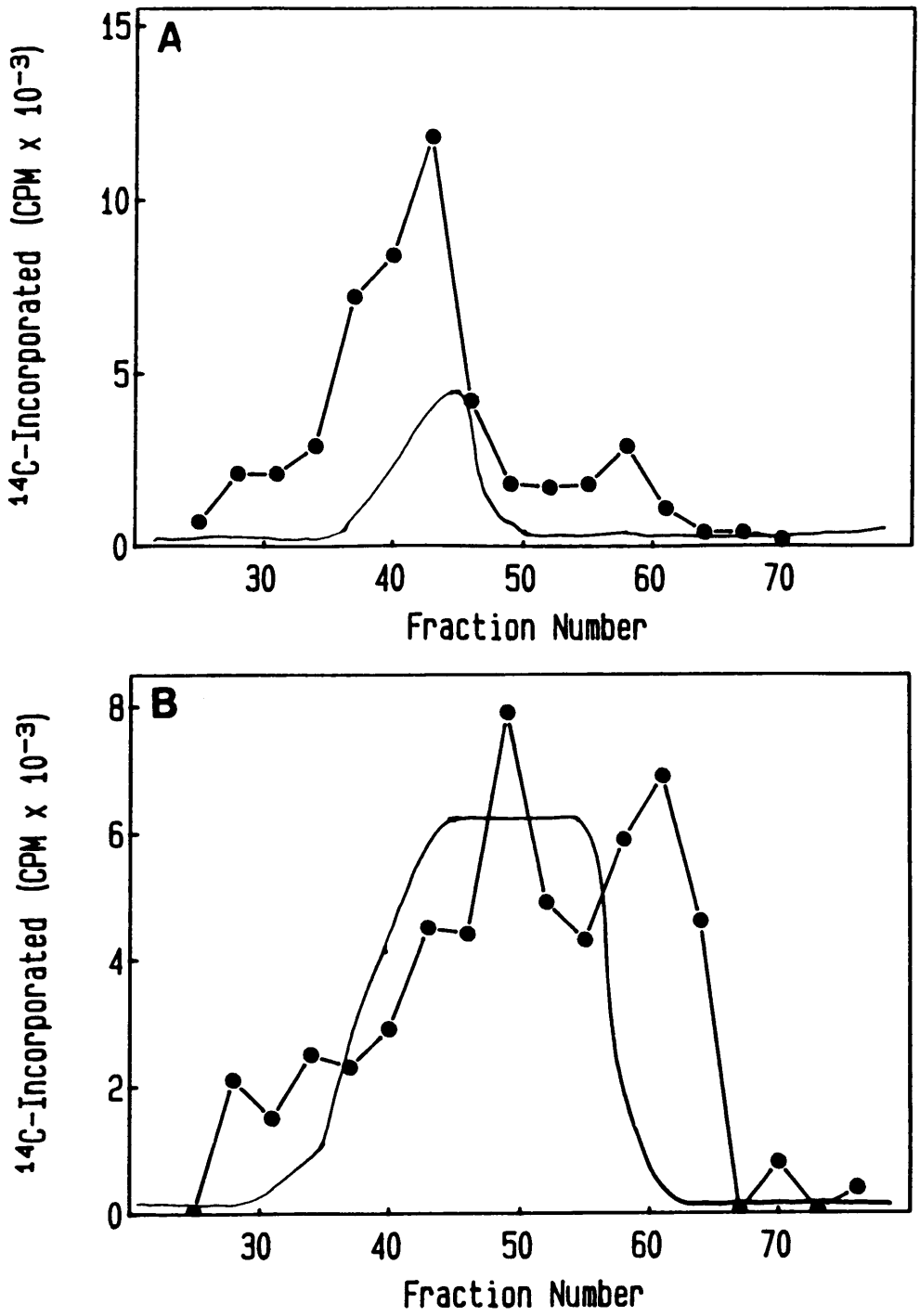

Figure 19. **S-300's of DE-Flow Throughs of NH_4SO_4 Precipitations:** A. The DE-Flow Through of the 10%-25% NH_4SO_4 pellet was NH_4SO_4 precipitated to 70% saturation, and the resulting pellet resuspended in 3 ml Buffer A containing 50mM KCl. The column was run at 30 ml/hr and 2 ml fractions were collected. Activity peaked at fraction 43 indicating a molecular weight of 200 kd for the holoenzyme. B. The same procedure as described above was used on the 25%-35% NH_4SO_4 DE-52 Flow-Through. The activity eluted as a broad peak due to the much greater amount of protein loaded as compared to panel A. The thin line in both panels represents the A_{280} at a scale of 0-1.0 Absorbance.

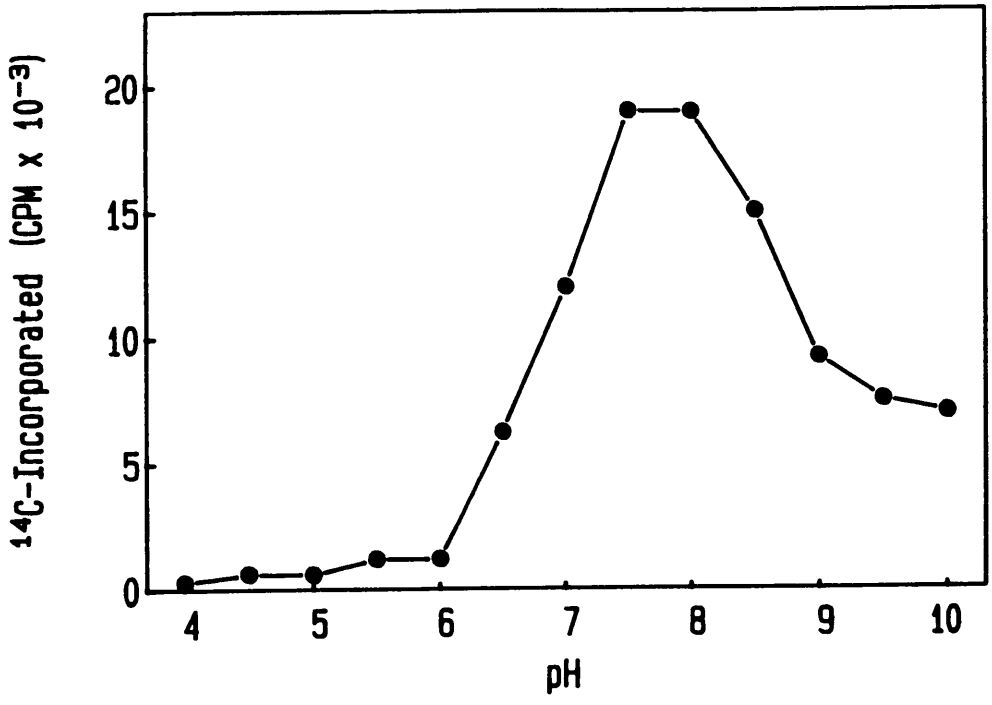

Figure 20. **pH Optimum of Partially Purified Glycogen Synthase I:** The pH optimum was determined by washing the glycogen synthase *I* into 10mM Tris-HCl pH 7.0 and mixing this 1:1 with 200 mM buffer at the desired pH. (Tris-HCl for pHs above 7 and Acetate for pHs below 7) Activity was measured in reaction mixtures made to the desired pH. The activity preferred a pH in the alkaline range peaking between pH 7.5-8.0 but did not lose much activity even at the higher pHs whereas at pH's below 6, it was inactive.

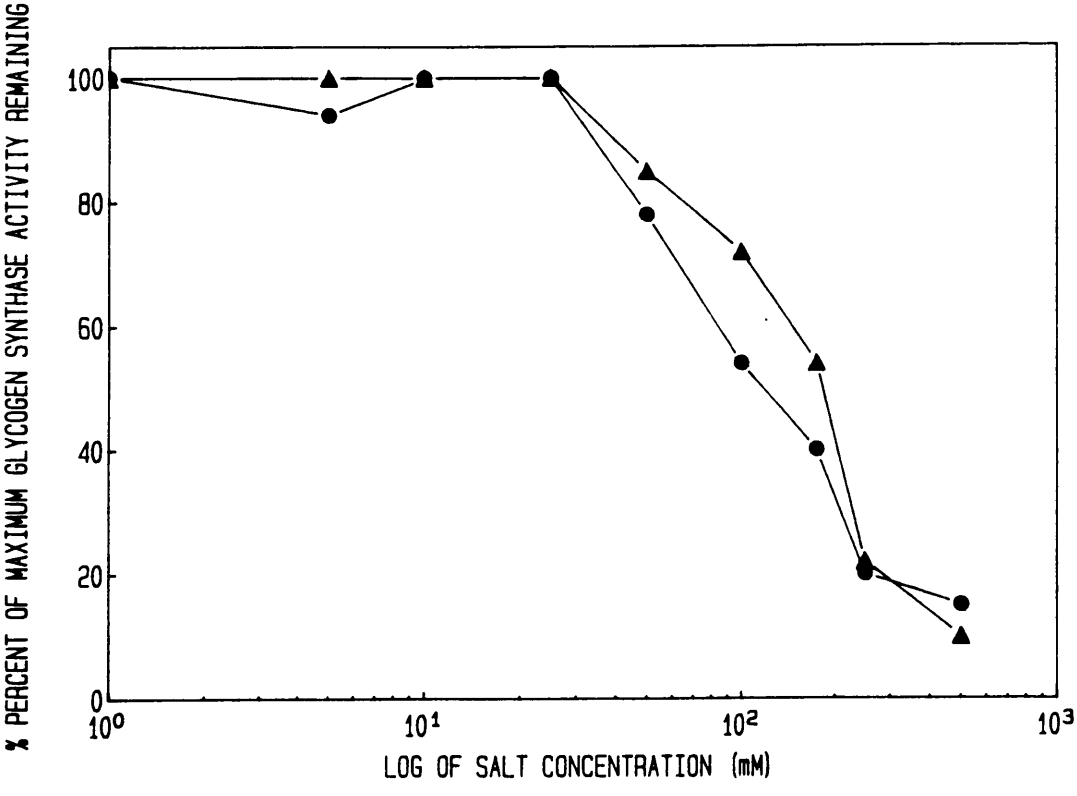

Figure 21. Salt Inhibition of Partiallly Purified Glycogen Synthase I: Glycogen synthase *I* was exposed to 0-500 mM KCL (▲) or NaCl (●) to observe the effect of salts on enzyme activity. Inhibition did not reach 50% of activity until ≃ 125 mM NaCl and 175 mM KCl. This corresponded to salt inhibition and not increase in ionic strength since increasing buffer ionic strength resulted in little activity loss to greater than 300 mM.

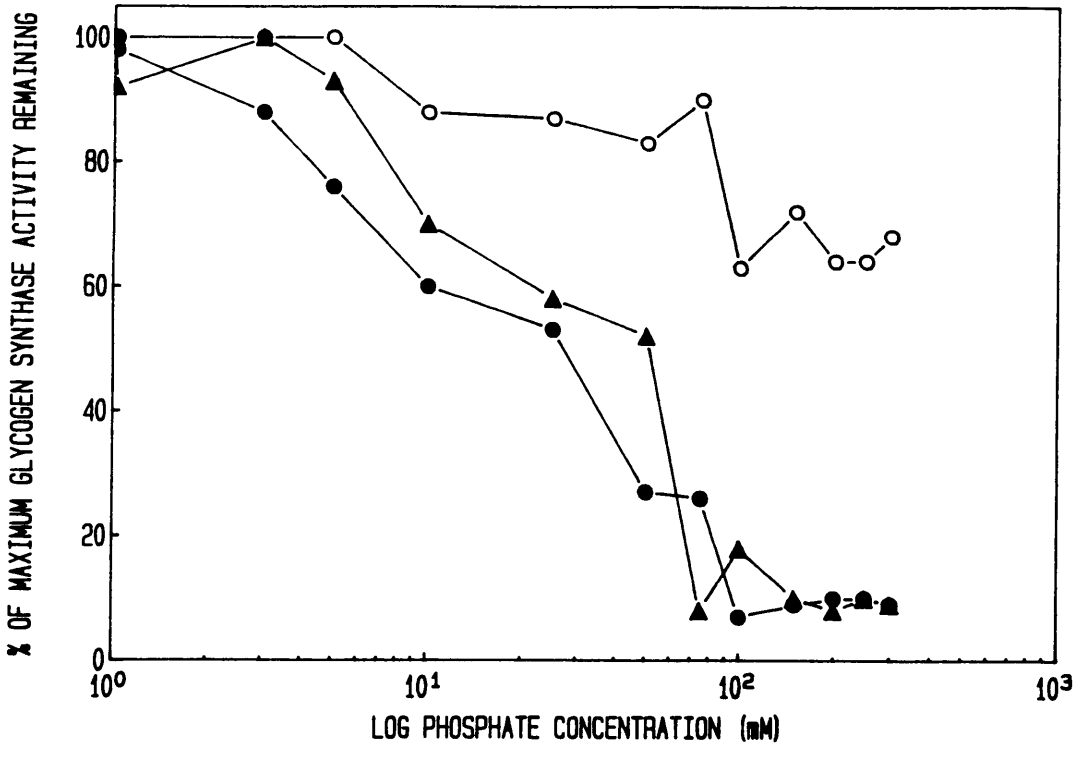

Figure 22. Inhibition of Glycogen Synthase we by Potassium Phosphate: Saunders and Wright (1977) had observed total inhibition of glycogen synthase *I* activity in 122,000 x g supernatants at low levels of phosphate. we found that this inhibition dissipates upon further purification. Shown here are the effects of phosphate at 0-300 mM concentrations on the activity of glycogen synthase *I* at different purification levels including 100,000 x g supernatant (●), 10-25% DE-52 Flow Through (▲) and 10%-25% S-300 pooled and conc. fractions (○). The 100,000 x g supernatant showed inhibition at low levels of phosphate, while the DE-52 Flow Through exhibited 50% inhibition at 50 mM phosphate and the pooled S-300 fractions didn't show inhibition above 35% at up to 300 mM phosphate. This may indicate phsophate inhibition acts through a protein component lost upon purification.

Glycogen synthase activity requires two components; glycogen is required as a primer for the addition of glucose units obtained from the second component, UDPG. In many cases of dual substrates, the Km of one substrate is dependent upon the concentration of the other substrate. Figures 23 and 24 illustrate this determination for the *Dictyostelium* glycogen synthase. Figure 23 shows the Km determination of glycogen synthase for UDPG at 4 concentrations of glycogen. The Kmapp for UDPG ranged from 2.8 mM at 5 mg/ml glycogen to 4.5 mM at 0.625 mg/ml glycogen. The Km for UDPG determined from these Kmapp was 2.4 mM. In Figure 24, the Km for glycogen was determined at 4 concentrations of UDPG. The Kmapp for glycogen ranged from 0.6 mg/ml at 4 mM UDPG to 0.95 mg/ml glycogen at 1 mM UDPG. The Km for glycogen determined from these Kmapp was 0.45 mg/ml. These Kms were comparable in their range to those determined for glycogen synthase from other systems. For example, the rabbit glycogen synthase Km value for UDPG determined by several researchers varied from 0.5 mM to 2.2 mM (Hizukuri and Larner 1964; Rosell-Perez 1962) while the rabbit muscle glycogen synthase Km for glycogen was determined by (Huang and Cabib 1974) to be 0.9 mg/ml.

4.3.3 Attempted Conversion of Glycogen Synthase I to D In Vitro

The similarities of glycogen synthase from *Dictyostelium* to the enzyme from muscle glycogen synthase were sufficient basis for investigating interconversion of the *I* to *D* forms. In rabbit muscle, glycogen synthase can be converted from the *I* form to the *D* form by dialysis of a muscle extract in the presence of Mg^{2+} (Friedman and Larner 1962). Apparently sufficient ATP is present to catalyze the conversion. Phosphatase inhibitors were added to force the reaction in the direction of phosphorylation. Extensive studies of rabbit muscle and liver glycogen synthase have shown its conversion to be catalyzed by cAMPdPK (Soderling et al. 1970; 1977) as well as other protein kinases including casein kinases and phosphorylase kinase (Huang et al. 1983; DePaoli-Roach et al. 1979). Table 3 illustrates some of our attempts at converting glycogen synthase *I* to the *D* form. Activity is shown as the percentage of the *I* form remaining after treat-

ment. Incorporation of ^{32}P into protein could not be used to determine if any phosphorylation was occurring since these were only partially purified samples and the subunit molecular weight of *Dictyostelium's* glycogen synthase has not yet been determined. Thus, a radioactive protein band could not be identified as glycogen synthase on an autoradiogram of an SDS-PAGE gel containing samples of reaction mixtures from Table 3. None of the treatments employed resulted in inhibition of glycogen synthase *I* activity that could be relieved by the addition of G-6-P. However, this lack of conversion of the two forms does not rule out phosphorylation as a method of regulating glycogen synthase activity. As can be seen from the initial investigations of rabbit muscle glycogen synthase, there were several factors which must be included in conversion reaction mixtures, and these factors may differ for the *Dictyostelium* enzyme.

4.3.4 Glycogen Synthase Activity During Growth and Development

Glycogen synthase *I* activity remained at a relatively constant level throughout growth (Table 4). Glycogen synthase *I* was found only in the cytoplasm, no activity was bound to the glycogen pellet.

Table 5 shows a representative experiment for glycogen synthase activity followed through the time course of development. Early in development all activity was bound to the glycogen pellet as the *D* form. After cell aggregation, the *I* form became the predominant form and all activity was found in the cytosolic fraction. The total activity remained fairly constant throughout development. Rosness et al. (1971) found glycogen synthase *I* activity to the predominant form early in development and the *D* form predominating by culmination and fruit. Our results were the exact opposite. There were several experiments where we did not observe glycogen synthase *D* at all during development but we never saw the *D* form activity late in development. Rosness did his work with the NC-4 strain (wild-type) strain while we used the Ax-3 strain. The observed differences in the appearance of the two forms of glycogen synthase may be due to some difference in the regulatory mechanisms controlling the appearance of glycogen synthase *D*. Although, such

differences in enzyme regulation have not been observed with previously characterized marker enzyme activities.

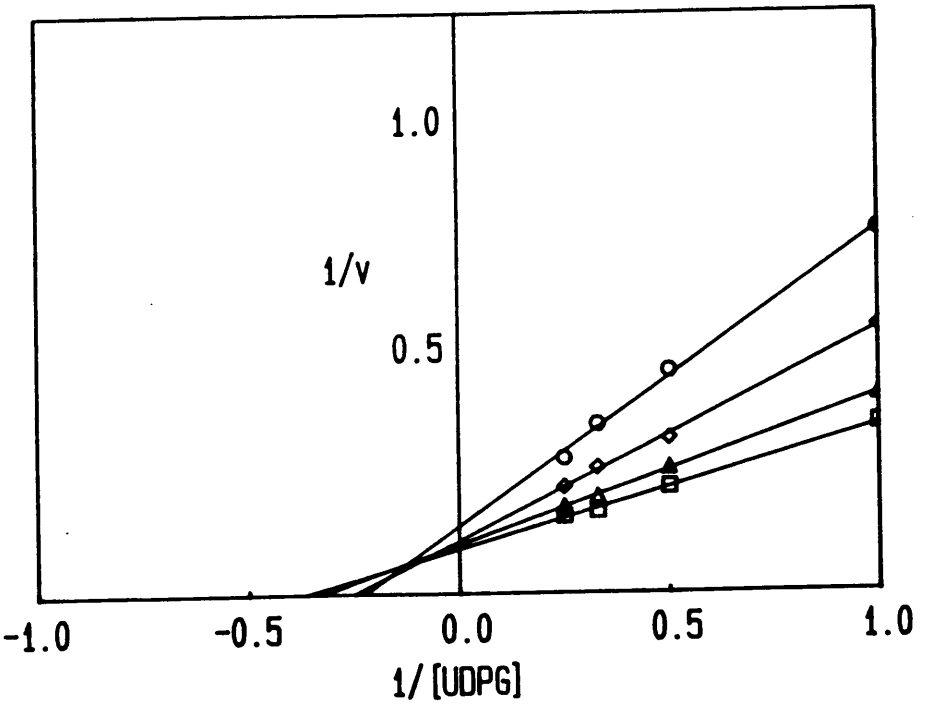

Figure 23. **UDPG Km Determination at varying Glycogen Concentrations:** The Km for UDPG was measured at 4 constant glycogen concentrations--5 mg/ml (□), 2.5 mg/ml (△), 1.25 mg/ml (◇), and 0.625 mg/ml (○). At each of these constant glycogen concentrations 4 UDPG concentrations--1 mM, 2 mM, 3 mM, and 4 mM. Their reciprocals were plotted as a LineWeaver-Burke plot and the Km was determined at each glycogen concentration. At 5 mg/ml glycogen-Km UDPG = 2.8 mM; at 2.5 mg/ml glycogen-Km UDPG = 3 mM; at 1.25 mg/ml glycogen-Km UDPG = 4 mM; and at .625 mg/ml glycogen-Km UDPG = 4.5 mM. The values showed no signs of becoming hyperbolic which occurred when Saunders and Wright (1977) measured these values for the *D* form. The Km for UDPG determined from these Kmapp was 2.4 mM.

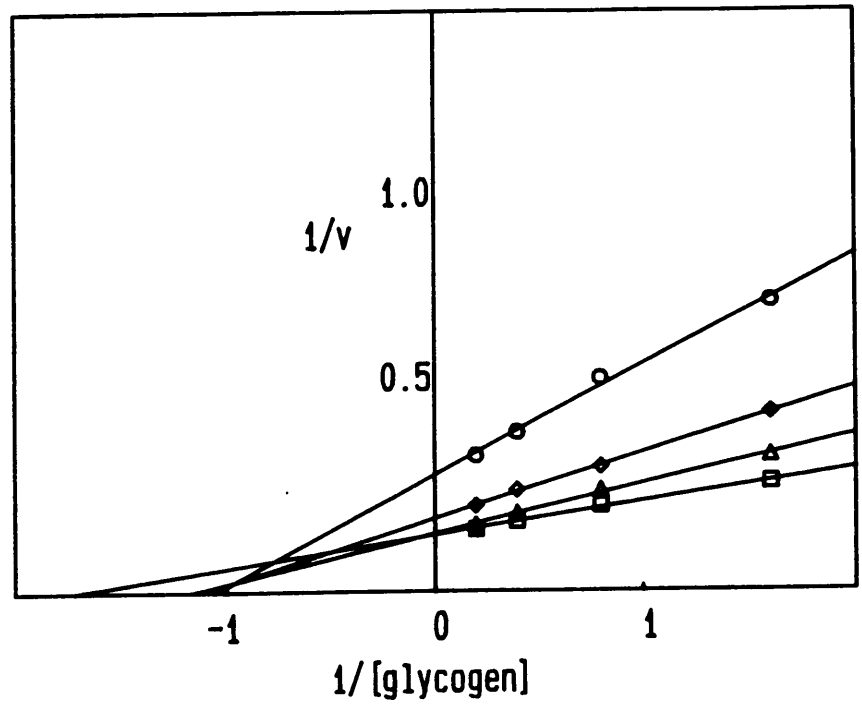

Figure 24. **Glycogen Km Determination at varying UDPG Concentrations:** As was done in Figure 23, the Km for glycogen was measured at 4 constant UDPG concentrations--4 mM (□), 3 mM (△), 2 mM (◇), and 1 mM (○). At each of these concentrations, 4 glycogen concentrations were used-- 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, and .625 mg/ml. The reciprocals were plotted as Lineweaver-Burke plots and the Kms determined at each UDPG concentration. At 4 mM UDPG--Km glycogen = 0.6 mg/ml; 3 mM UDPG--Km glycogen = 0.8 mg/ml; 2 mM UDPG--Km = 0.9 mg/ml; 1 mM UDPG--Km glycogen = 0.95 mg/ml. The Km for glycogen determined from these Kmapp was 0.45 mg/ml.

Table 3. Attempted in vitro Conversions of Glycogen Synthase I to Glycogen Synthase D

Conditions[1]	Percent Independent Activity[5]
Control (No additions)	97
5 mM Mg^{2+}	96
500 uM ATP	96
5 mM Mg^{2+} + 500 uM ATP	95
Mg^{2+}-ATP + cAMPdPK[2]	97
Mg^{2+}-ATP + Phosphatase Inhibitors[3]	97
Mg^{2+}-ATP + Protease Inhibitors[4]	96
Mg^{2+}-ATP + Phosphatase Inhibitors + Protease Inhibitors + cAMPdPK	95
Mg^{2+}-ATP + Phosphatase Inhibitors + Protease Inhibitors + cAMPdPK at 4 C	94
Mn^{2+}-ATP + Phosphatase Inhibitors	96

[1]Glycogen Synthase I in 8000 x g supt. and 100,000 x g supt. from amoeba, early aggregation, and slug were used with much the same results. Enzyme samples were assayed for 15 min after preincubation and a 1:10 dilution of the enzyme sample.

[2]The cAMPdPK was from a peak fraction off an S-300 gel filtration column.

[3]Phosphatase inhibitors were 20 mM NaF and 10 mM sodium pyrophosphate.

[4]Protease Inhibitors were 1 ug/ml Antipain, 10 mM PMSF, 5 mM benzamidine, and 1 ug TPCK.

[5]Total activity decrease from 10%-30% during incubation but no change G-6-P dependency was observed.

Table 4. Glycogen Synthase Activity During Growth

Cell Concentration[1] (cells/ml)	Glycogen Synthase Activity (nmol/min/mg wet weight)	
	Experiment 1	Experiment 2
1.7×10^6	32 ± 0.15	31 ± 0.6
4×10^6	38 ± 0.5	37 ± 0.3
8×10^6	24 ± 6.15	30 ± 1.4
1×10^7	32 ± 0.3	32 ± 0.15

[1]Cell concentration determined using a hemacytometer.
[2]Activity is the average of duplicates + Standard Error of the Mean. Samples were 8000 x g supt. diluted 1:50 in Buffer A. Activity was assayed +/- G-6-P but no glycogen synthase D activity was observed.

Table 5. Glycogen Synthase Activity During Development

Stage of Development[1]	Glycogen Synthase Activity[2] (nmol/min/mg wet weight)			
	Supernatant	Percent I Activity	Pellet	Percent I Activity
Amoeba	-	-	100 ± 1.3	14
Early Aggregate	-	-	180 ± 10	11
Tight Aggregate	190 ± 2.3	76	-	-
Early Slug	90 ± 9.1	81	-	-
Early Culmination	120 ± 1.1	92	-	-
Fruit	160 ± 4.2	96	-	-

[1]Stage of development was determined by visual characteristics. The amoeba stage had been on membranes for 1 hr prior to harvesting. Some asynchronicity was observed at culmination and fruit.

[2]Activity is the average of duplicates + standard error of the mean. Samples were 100,000 x g supt. and pellets diluted 1:25 in Buffer A.

4.4 Discussion

Although this study does not provide evidence in favor of glycogen synthase's cAMP-dependent phosphorylation, it does provide a basis for further clarification of the enzyme's regulation. A major obstacle in clarifying its regulation is the difficulty in purifying either form from *Dictyostelium.* Our attempts to stabilize the activity by the addition of glycerol, glycogen, salt, or the addition of a cocktail of protease inhibitors were unsuccessful. The inability of the enzyme to bind to anion or cation exchange columns within its stability range may indicate a hydrophobic molecule. With the rabbit muscle and liver glycogen synthase, hydrophobic chromatography (γ-aminobutyl agarose) and glycogen sepharose affinity chromatography have been used to purify glycogen synthase (Huang et al. 1983). However, the hydrophobicity must be qualitatively different in *Dictyostelium* since although the *I* form uses glycogen as a primer, it does not bind to the glycogen pellet as does the *D* form. Nor were we able to purify the glycogen synthase *I* using a glucosamine-6-phosphate affinity column which has been used to purify both forms from rabbit muscle (Soderling et al. 1977).

The properties of glycogen synthase which could be determined were comparable to previously described glycogen synthase purified from a variety of sources (Takeda et al. 1975). The salt and phosphate inhibition studies did not provide any clue to the loss of activity upon further purification. Although a cocktail of protease inhibitors was ineffective in maintaining activity, the loss of enzyme activity may yet be due to proteases unaffected by the protease inhibitors used. *Dictyostelium* is known to have several proteases which are not effectively inhibited by protease inhibitors that are highly effective against mammalian proteases (North and Harwood 1979).

Only recently Rutherford and Cloutier (1987) described two forms of glycogen phosphorylase, the glycogen degradative enzyme, in *Dictyostelium.* This enzyme is found as a 5'-AMP dependent form early in development (*b* form) and as a 5'-AMP independent form after aggregation. In eucaryotes, where two forms of the phosphorylase are found, the two forms are interconvertible by a phosphorylation-dephosphorylation cycle. It is yet to be determined in

Dictyostelium whether phosphorylase is regulated in this manner. However, it seems likely that the regulation of glycogen synthase and glycogen phosphorylase whether through a phosphorylation event or through regulation of gene activity, would be similar. The similarity of the allosteric regulation of glycogen synthase and glycogen phosphorylase to the allosteric regulation of their mammalian counterparts lends support to regulation by a phosphorylation event. However, the unique positions of glycogen phosphorylase and glycogen synthase in the cellular differentiation of *Dictyostelium* may well indicate regulation at the level of the gene. Further investigation both at the level of the enzyme and at the level of the gene should clarify the regulatory mechanism.

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