A key step in the cellular differentiation of Dictyostelium is the degradation of glycogen to provide the precursors for synthesis of the structural end products of development. We have found that the enzyme that initiates this degradative pathway, glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1), is developmentally regulated and exists as two forms. During the time course of development, a previously undescribed activity, the "b" form, decreases, whereas that of the "a" form increases. The b form is inactive unless 5'-AMP is included in the reaction mixture. The mechanism of activation by 5'-AMP is by a 40-fold increase in the affinity of the phosphorylase for its substrates. Both forms were purified to homogeneity. They have identical subunit molecular weights of 90,000 and both exist as a dimer under non-denaturing conditions. The two forms are also identical with respect to salt inhibition, optimum temperature for activity, and pH optimum. They differ in their elution from DE52-cellulose, affinity constants, thermal stability, affinity for 5'-AMP-Sepharose, and their peptide maps. Attempts to demonstrate interconversion of the two activities by a kinase-directed phosphorylation have been unsuccessful. We report here the existence, the developmental regulation, the purification to homogeneity, and some of the physical and kinetic properties of both the 5'-AMP-dependent and -independent forms of the enzyme.

The development program of Dictyostelium discoideum has been, and continues to be, exploited as an easily manipulated model system for the study of cellular differentiation. Differentiation can be induced by transfer of single-celled amoebae to a non-nutrient medium. Certain cells in the population spontaneously begin to secrete cAMP in a pulsatile manner. In addition to acting as a chemotactic signal towards which the remaining cells aggregate forming a multicellular structure called the slug. After a period of migration, the cells within the slug undergo terminal differentiation resulting in two cell types providing a cellulosic stalk supporting an aerial spore mass.

In addition to acting as a chemotactic signal early in Dictyostelium development, cAMP has been shown to regulate the activity of a cAMP-dependent protein kinase (1-6). Cyclic AMP-dependent phosphorylase of endogenous proteins has also been demonstrated (7). The question then becomes, "What enzyme systems might be developmentally regulated via a cAMP-dependent phosphorylation-dephosphorylation cascade?" The classic example in mammalian systems is that of glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase; EC 2.4.1.1); indeed, it was the discovery of the regulation of this enzyme via a phosphorylation-dephosphorylation mechanism in rabbit muscle which led to the discovery of cAMP-dependent protein kinase.

Glycogen degradation in D. discoideum is developmentally regulated (8, 9). Glycogen levels increase during the first 18 h of development from nearly undetectable concentrations to a maximum value at the early culmination stage. Certain cells at this stage migrate to a position within the individual where a cellular stalk is being constructed. Upon reaching this position of stalk construction, the cellular glycogen is degraded and the resulting glucose units are used for synthesis of the stalk cellulose (10). As the stalk is synthesized the remaining prespore cells are lifted off the substratum. Upon completion of the stalk the prespore cells rapidly degrade their glycogen and construct a cellulosed-walled spore (10). Thus glycogen is not used as an energy reserve as it is in most organisms, but instead as a source of precursors for the synthesis of the end product of development (11-13). Previous investigators have reported only one form of glycogen phosphorylase in Dictyostelium and suggested that the enzyme was similar to that found in plants (14-18). They found that a 5'-AMP-independent activity increased during development and that the mechanism of this increase was by de novo synthesis of enzyme protein. In this paper we report on the existence, the developmental regulation, the purification to homogeneity, and some of the physical and kinetic characteristics of both a 5'-AMP-activated "b" form and a 5'-AMP-independent "a" form of glycogen phosphorylase.

**Materials and Methods**

Preparation of Cell-free Extracts-Growth and differentiation of D. discoideum (Ax3) was carried out as described previously (19). For purification of the 5 form of the enzyme, stationary phase amoebas (70-120 g, wet weight) were washed free of nutrient medium and resuspended in 5 volumes of 50 mM Tris-HCl (pH 7.5) containing 0.02% sodium azide, 2 mM mercaptoethanol, and 2 mM benazidine (buffer A). The cells were ruptured by three 45-s exposures to a 2-cm probe of a sonic cell disruptor (model 300, Fisher) at a setting of 45. The sonicate was then centrifuged at 100,000 x g for 1 h.

For purification of the a form of the enzyme, cells at the culmination stage of development (70-120 g, wet weight) were washed from an agar surface with 2 mM EDTA, 14 mM mercaptoethanol (pH 7.0). After washing once with this solution the cell pellet was frozen at -70°C. The cells were thawed by resuspending them in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 14 mM mercaptoethanol and centrifuged at 100,000 x g for 60 min.

**Purification of Phosphorylase a—A 100,000 x g supernatant from culmination stage cells was brought to a final concentration of 2.0%,
w/v, with solid streptomycin sulfate and stirred for 30 min at 4°C. After removing the nucleic acids by centrifugation at 20,000 × g for 40 min and dialyzed overnight against 4 liters of 10 mM potassium phosphate buffer (pH 7.0) containing 14 mM mercaptoethanol, the dialyzed sample was centrifuged to remove any insoluble material and applied to a DE52-cellulose column (1.9 × 86 cm) that had been equilibrated in 25 mM potassium phosphate buffer containing 14 mM mercaptoethanol. The material that did not bind to the column was allowed to elute completely as determined by the return to the baseline on a column monitor. Proteins that were bound to the column were then eluted for 8 h with a linear 0–1 M KC1 gradient in 25 mM potassium phosphate buffer (pH 7.0) containing 14 mM mercaptoethanol (flow rate, 80 ml/h; 6-min fractions; total volume of gradient was 640 ml) as formed by a programmable pump (ISCO model 382). The activity was eluted at approximately 50 mM potassium chloride, in a total volume of approximately 100 ml. The pooled DE52-cellulose fraction column was brought to 70% saturation with solid ammonium sulfate, stirred 30 min, and centrifuged at 20,000 × g for 20 min. The supernatant was then dialyzed for 1 h against 2 liters of water and then for 2 h against 2 liters of 10 mM buffer A. After removing any insoluble material that formed during dialysis, the supernatant was applied to an aminoethylagarose column (1.6 × 7 cm) that had been equilibrated in 10 mM buffer A. The flow-through material was collected and the column was eluted for 8 h with a linear 0–50 mM potassium phosphate gradient in 10 mM buffer A (flow rate, 80 ml/h; 6-min fractions; total volume of gradient was 640 ml). The pH was held constant at 7.5. The phosphorylase activity eluted at approximately 30 mM potassium phosphate. The active fractions from the aminoethylagarose column were pooled (approximately 80 ml) and concentrated to 2 ml in a stirred filtration cell (Amicon YM-10 membrane). The sample was then applied to a Sephacryl S-300 gel filtration column (1.6 × 86 cm) that had been equilibrated in buffer A containing 100 mM NaCl. The activity eluted as a symmetrical peak that was coincident with a protein peak corresponding to a molecular weight of 180,000.

**Purification of Phosphorylase b**—The 100,000 × g supernatant from stationary phase amoebas was added to approximately 50 ml of DE52-cellulose that had been equilibrated with buffer A. After brief mixing the entire slurry was poured into a column (3 × 5 cm). The material that did not bind to the resin was allowed to elute, as indicated by a column monitor. Protein that was bound to the resin was eluted for 8 h with a linear 0–0.05 M potassium phosphate gradient in 50 mM buffer A (flow rate, 80 ml/h; 6-min fractions; total volume of gradient was 640 ml). The pH was held constant at 7.5. The phosphorylase activity eluted at approximately 30 mM potassium phosphate. The active fractions from the aminoethylagarose column were pooled (approximately 80 ml) and concentrated to 2 ml in a stirred filtration cell (Amicon YM-10 membrane). The sample was then applied to a Sephacryl S-300 gel filtration column (1.6 × 86 cm) that had been equilibrated in buffer A containing 100 mM NaCl. The activity eluted as a symmetrical peak that was coincident with a protein peak corresponding to a molecular weight of 180,000.

**Gel Electrophoresis—Analytical PAGE in the presence of SDS was as described by Laemmli (20). The gels were either silver-stained (21) or were Coomassie-stained (22). For preparative SDS-PAGE, 1 ml of the enzyme sample was applied to a single large well at the top of the stacking gel. After electrophoresis at 40 mA the protein was visualized by either Coomassie Blue staining, or by placing the gel in 4 mM sodium acetate (18). In the latter case the protein appeared as a clear band against an opaque background. The protein band was cut from the gel using a new razor blade, rinsed in water, and then frozen at −70°C. The gel slices were thawed by placing them in 40 mM Tris-HCl (pH 8.6) containing 0.01% SDS for 20 min at room temperature. The gel slice was then removed from the buffer, minced with a razor blade, and placed in a large sample well of an electrophoretic eluter (ISCO model 1750). The protein was eluted from the gel in the same buffer for 4 h at room temperature at a power setting of 1 watt.

**Peptide Mapping**—The purified a form of the enzyme, as obtained from the Sephacryl S-300 column, or the purified b form, as eluted from the 5'-AMP-Sepharose column (see Table I), was concentrated to 1 ml and subjected to preparative SDS-PAGE as described above. The gel slice containing the phosphorylase was removed and frozen at −70°C. Two procedures were used for proteolytic degradation of the electrophoretically pure peptide (23), with identical results. In one case the peptide was electrodialyzed from the gel into a given buffer. After incubation with a specific protease, a sample was removed and subjected to analytical SDS-PAGE (15%) gel as described above. This procedure was useful to follow the time course of proteolysis, the effect of different incubation buffers and pH, the peptide map resulting from degradation of the phosphorylase by different proteases, and the peptide map resulting from different amounts of the same protease. In the latter two cases we were able to comigrate peptide maps of the two forms of the enzyme, in which complete proteolytic cleavage had occurred. We found conditions, for example, where increasing the amount of a specific protease in the reaction mixture did not change the peptide map, and the amount of time that was required for a protease to produce a given set of degradation products. Thus any differences in the peptide maps between the two forms of the enzyme were not due to incomplete degradation of one or the other of the proteins.

In the second procedure for peptide mapping, individual bands from the preparative gel (7.5% gel), were cut out with a razor blade and directly lyophilized. The gel was then photographed immediately after the gel was removed from the gel, and the gel slice was then removed from the preparative gel (7.5% gel) and placed directly in the sample well of a second analytical SDS-PAGE (15%) gel (approximately 3 × 5 mm). The peptide slice was then overlaid with various amounts of a protease in 10 μl of the buffer to be tested containing 10% glycerol. In initial experiments the current was turned off for 15 min after the tracking dye reached the interface between the stacking gel and the separating gel, as suggested by Cleveland et al. (23). We found, however, that this was not necessary since identical peptide maps were obtained when the current was not turned off. The gels were stained with either silver (21) or Coomassie Blue (22).

**RESULTS**

**Demonstration of Two Forms of Phosphorylase and Purification of Both Forms to Homogeneity**—When a 100,000 × g supernatant from early slug stage cells was loaded onto a DE52-cellulose column at pH 7.5 and eluted with a linear KC1 gradient, two distinct glycogen phosphorylase activities were eluted as illustrated in Fig. 1. The first activity to elute from the column (approximately 50 mM KC1) was dependent on 5'-AMP activity, whereas the later eluting peak (approximately 180 mM KC1) was almost completely dependent on 5'-AMP for activity. We will refer to these proteins as the a and b forms, respectively, since this is the designation used on other systems for 5'-AMP-independent and -dependent activities. Extracts prepared from undifferentiated vegetative amoebas contained only the b form, whereas extracts from a late stage of development, the culminating stage (approximately 18 h of development), contained primarily the a form. The stage of development shown in Fig. 1 is intermediate between the amoebas and culminating stages, and thus both a and b forms are present.

A glycogen phosphorylase that appears during the culminating stage of development has been purified to homogeneity by Thomas and Wright (16) and by Higgins and Dahmus (18). This enzyme is undoubtedly the 5'-AMP-independent a form as described here. In our hands neither of the purification...
Glycogen Phosphorylase in Dictyostelium

![DE-52 Cellulose](image)

**FIG. 1.** DE52-cellulose chromatography of glycogen phosphorylase at the early slug stage of development. The 100,000 X g supernatant was mixed with 50 ml of DE52-cellulose in 50 mM buffer A at pH 7.5. Enzyme was eluted from the column (3 X 5 cm) with a linear gradient of 0-0.2 M KCl. No phosphorylase activity was found in the flow-through volume (not shown). Fraction number one is the beginning of the salt gradient. Column fractions were assayed as described under “Materials and Methods.” Glycogen phosphorylase activity was assayed with (C) and without (A) 3 mM 5'-AMP.

The procedure that was used for the a form, because the activity was assayed with 5'-AMP, and without (A) 3 mM 5'-AMP.

| Fraction | Volume (ml) | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification fold (X) | Yield (%) |
|----------|-------------|--------------|-----------------------|-----------------------------|-----------------------|-----------|
| I. a form |             |              |                       |                             |                       |           |
| 1. 100,000 X g supernatant | 350 | 2030 | 46.0 | 0.023 | 1 | 100 |
| 2. Streptomycin sulfate supernatant | 350 | 1330 | 46.0 | 0.035 | 1.5 | 100 |
| 3. Ammonium sulfate 35-65% pellet | 16 | 740 | 32.0 | 0.043 | 1.9 | 70 |
| 4. 50 °C treatment | 11 | 343 | 20.0 | 0.082 | 3.6 | 61 |
| 5. DE52-cellulose | 120 | 13.6 | 7.0 | 0.51 | 22.2 | 15 |
| 6. Aminobutyrl-agarose | 80 | 2.0 | 3.5 | 1.75 | 76.1 | 76.1 |
| 7. Sephacryl S-300 | 0.9 | 0.23 | 1.0 | 4.3 | 187 | 2.2 |

II. b form

| Fraction | Volume (ml) | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification fold (X) | Yield (%) |
|----------|-------------|--------------|-----------------------|-----------------------------|-----------------------|-----------|
| 1. 100,000 X g supernatant | 412 | 2595 | 60.0 | 0.023 | 1 | 100 |
| 2. DE52-cellulose | 280 | 221 | 13.0 | 0.059 | 2.6 | 22 |
| 3. 5'-AMP-Sepharose | 14 | 3.0 | 10 | 3.3 | 143 | 17 |

In these extracts in which the two forms of the enzyme are known to be phosphorylated and dephosphorylated forms of the same gene product, both the a and b activities bind to the 5'-AMP agarose.

Higgins and Dahmus (18) showed immunological cross-reactivity between their purified M, 90,000 protein and other peptides at M, 101,000 and 105,000. They suggested that the M, 90,000 peptide was a degradation product of the higher molecular weight forms. We, therefore, tested the effect of including a number of different protease inhibitors in the purification procedure of both forms of the enzyme (phenylmethylsulfonyl fluoride, 170 μg/ml; N-α-p-tosyl-L-lysine chloromethyl ketone, 18 μg/ml; benzamidine, 300 μg/ml; N-tosyl-L-phenylalanine chloromethyl ketone, 17 μg/ml; pepstatin A, 1 μg/ml), but found no effect of these inhibitors on the molecular weight of the phosphorylases.

We found that the specific activity of the Dictyostelium phosphorylase in freshly prepared cell-free extracts was remarkably constant under a variety of conditions. For example, the specific activity of the a and b forms from 100,000 X g supernatants of late and early stages, respectively, were identical—0.023 μmol/min·mg of protein. This value was highly reproducible in over 25 attempts to develop a purification procedure for the enzyme. This specific activity is also quite similar to that obtained by Higgins and Dahmus (18) of 0.035 unit/mg and by Thomas and Wright (16) of 0.032 unit/mg. In both of these reports the activity of phosphorylase was not assayed in the presence of 5'-AMP and, therefore, undoubtedly represents the a form of the enzyme as described here.

We also found that the a and b forms were present in both the wild-type strain of D. discoideum (NC4) and in the axenic strain (Ax3), and that the specific activity was the same for both strains. In fact, when lyophilized samples of the wild-type strain that had been stored for 5 years at −70 °C were reconstituted and assayed for phosphorylase, the specific activity was identical to that of freshly prepared extracts. We also followed the specific activity of the b form during the growth phase of Ax3 amoebae. A constant level of activity was found during early, middle, and late logarithmic growth as well as over the course of 5 days of stationary phase growth. It should also be pointed out that the specific activity of the purified a and b forms are approximately the same (Table I).

In a number of systems the phosphorylase activity remains associated with cellular glycogen during subcellular fractionation. On the contrary, the Dictyostelium enzyme was always found in the soluble fraction, not associated with the glycogen.
fraction. The cells were ruptured by a number of methods, including freeze-thaw, sonication, French pressure cell, and lyophilization, and in each case the enzyme was found in the soluble fraction regardless of the method of cell rupture.

Properties of the Two Forms of the Enzyme—Fig. 2 illustrates the glycogen phosphorylase a elution profile from a Sephacryl S-300 gel filtration column. The enzyme eluted in fractions corresponding to a molecular weight of \( \sim 180,000 \). Both the 5'-AMP-dependent and -independent enzymes, when applied to the column individually, gave identical elution profiles. SDS-PAGE analysis of both forms showed subunit molecular weights of 90,000. Thus, under the conditions in which the enzymes were applied to the column, both forms exist as dimers.

The effect of high salt on enzyme activity is illustrated in Fig. 3. Both forms of the enzyme displayed 50% inhibition in the presence of 80 mM KCl. Because of this strong inhibition by salt, all preparations of the enzymes that were to be used for the study of the properties of the two forms were desalted by elution from PD-10 columns (Pharmacia).

Both enzyme activities displayed a similar behavior when assayed at various temperatures from 10 to 42 °C. The data illustrated in Fig. 4 are a composite of several data points obtained with at least two different enzyme preparations of both the a and b enzyme activities. An incubation temperature of 28 °C provided the maximum activity. Although no difference was observed between the two forms with respect to the optimum temperature for assay, Fig. 5 illustrates the greater thermal stability of the 5'-AMP-independent enzyme. Previous investigators (17) found that the glycogen phosphorylase from the culmination stage was stable to heating at 50 °C for 5 min and therefore incorporated this step into their initial purification scheme. We found that the vegetative cell b enzyme was completely inactivated by such a heat treatment. Further evidence of the thermal instability of the b enzyme was found when cells harvested from the slug stage were lysed by mild sonication as compared to a freeze-thaw cycle. After sonication, both forms of the enzyme were detected, whereas in cells lysed by a freeze-thaw cycle, no b activity could be detected (25). We also found that the b activity was more labile than was the a form in crude extracts. When 100,000 \( \times \) g supernatants of amoeba (b form) and culmination stage (a form) were incubated for 48 h at 7 °C, the a activity was completely recovered, whereas the b activity was undetectable. The lack of detectable b enzyme in a number of reports from the literature can, therefore, be explained by the effect of freeze-thaw and/or heat treatments.

The two enzymes exhibited slightly different pH optima, as illustrated in Fig. 6. Over the range of pH values for which citrate and triethanolamine are effective buffers, neither was effective in illustrating a clear pH optimum. However, in the systems buffered with imidazole or MES, the phosphorylase a enzyme demonstrated a preference for a pH slightly below 6.6 (Fig. 6A), whereas the b enzyme preferred a pH slightly in excess of 6.6 (Fig. 6B).

The 5'-AMP-dependent enzyme displayed half-maximal activation with \( \sim 700 \mu M \) 5'-AMP (Fig. 7). The 5'-AMP \( K_a \)
Glycogen Phosphorylase in Dictyostelium

**FIG. 5. Thermal stability of the 5'-AMP-dependent and -independent glycogen phosphorylase enzymes.** Glycogen phosphorylase activity followed a preincubation of the independent α enzyme at 50°C (O), 55°C (○), 60°C (△), and 70°C (●), and the dependent β enzyme at 50°C (●) for the indicated times is shown. Inset gives the time required to reduce the enzyme activity by 50% at each of the indicated temperatures.

did not change with 2- and 4-fold dilutions of the enzyme. These data also provide an activity ratio (activity in the absence of 5'-AMP/activity in the presence of 5'-AMP) of ~0.08. The activity ratio in the presence and absence of 5'-AMP was not affected by treatment of the enzyme with charcoal or by passing the enzyme through a Sephadex G-25 gel filtration column. Thus the activity of the b form in the absence of 5'-AMP was not due to loosely bound 5'-AMP. Maximum activation was consistently achieved with 3 mM 5'-AMP.

The inorganic phosphate (P_i) and glycogen K_a values for both forms of the enzyme are shown in Fig. 8. The 5'-AMP-dependent enzyme had a P_i of 55 mM and a glycogen K_a of 150 mg/ml in the absence of 5'-AMP. The enzyme's affinity for both substrates was increased 36-fold in the presence of 5'-AMP, to 1.5 mM (P_i K_a) and 4.2 mg/ml (glycogen K_a) (Fig. 8, A and C, respectively). The 5'-AMP-independent enzyme has a P_i K_a of 1.2 mM and a glycogen K_a of 2.7 mg/ml (Fig. 8, B and D, respectively); this is 45- and 55-fold greater affinity, respectively, than the non-activated 5'-AMP-dependent enzyme. Thus, the increased activity of the a form over that of the b form in the presence of 5'-AMP compared to the absence of 5'-AMP, is due to the magnitude of the difference in their affinity for both substrates. The activity of the independent enzyme was not increased by the addition of 5'-AMP. In fact, levels of 5'-AMP which stimulated the dependent enzyme had a mild inhibitory effect on the activity of the independent enzyme (data not shown).

Both enzymes, under conditions providing maximal activity, display a similar response to varying concentrations of one substrate while holding the other constant. At decreasing concentrations of one substrate, the K_a of the other substrate increases, i.e., the enzyme's affinity for that substrate decreases (Fig. 9). Both enzymes showed substrate inhibition in response to increased phosphate concentrations (>10 mM) but not to glycogen (data not shown).

**Peptide Mapping of the a and b Forms**—In order to determine the structural similarity of the two forms of the enzyme we subjected the electrophoretically pure preparations to peptide mapping (Fig. 10). The procedure was done under a variety of conditions in order to rule out differential degradation of the two forms that might be due to the experimental procedure. For example, four different proteases were used, Staphylococcus aureus V8, chymotrypsin, trypsin, and papain; each was tested over a concentration range of 5 ng to 10 μg and each was tested over a pH range of 6.8-8.9, with or without EDTA and/or SDS. In addition, two types of incubation procedures were used. In one case the purified Dictyostelium phosphorylase was simply cut from a preparative SDS gel, and a small section of the gel was placed into the sample well of another gel. The protease was then layered over the gel sample and the current turned on. Thus degradation occurred in the stacking gel. The second procedure involved electroeluting the phosphorylase from the gel sample into an appropriate buffer. The protease to be tested was then preincubated with the phosphorylase in a given buffer and at a specific pH. After incubation for various lengths of time a sample was removed and subjected to SDS-PAGE (see "Materials and Methods").

Fig. 10 shows that proteolytic degradation of the a and b
forms by S. aureus V8 results in distinctly different peptide maps. Chymotrypsin and trypsin treatment also gave different peptide maps for the two forms of the Dictyostelium enzyme. Likewise, distinct maps for the two forms were obtained regardless of the pH used, the buffer used, or whether the incubation was in solution or directly in the gel, as described above. In fact, in over 25 experiments with various combinations of conditions, in no case were the peptide maps of the two forms the same. On the other hand, identical peptide maps were obtained in control experiments in which commercial rabbit muscle phosphorylase a and b were mapped.

**DISCUSSION**

In keeping with the established convention of glycogen phosphorylase nomenclature, the 5′-AMP-activated enzyme is designated here as the b form, with the a form being the nondependent form. This designation of the Dictyostelium enzyme is not intended to suggest that the two activities are phosphorylated and dephosphorylated forms of the same protein. Instead, the physical and kinetic properties presented in this report relate the degree of similarity between the Dictyostelium enzyme and those of previously described systems (26–30). We show that, during development, the activity of the a form decreased and that of the b form increased. The maximum specific activity of the two forms are equal (when the a activity is assayed in the presence of 5′-AMP). The b form is nearly undetectable unless 5′-AMP is included in the reaction mixture. The mechanism of activation of the b form by 5′-AMP is through a 36-fold increase in the affinity of the phosphorylase for its substrates. Both forms of the enzyme have the same subunit molecular weight of 90,000 and both exist as a dimer under non-denaturing conditions. The two forms are also identical with respect to salt inhibition and optimum temperature for activity. They differ with respect to their elution from DE52-cellulose, affinity constants, thermal stability and stability in crude extracts, affinity for 5′-AMP-Sepharose, and peptide maps.

Both the a and b enzymes showed marked inhibition by high salt (Fig. 3) and an incubation temperature optimum of 26°C (Fig. 4). This is slightly higher than the optimum temperature for growth and development, i.e., 23°C, and so may not be physiologically significant. The pronounced difference in the relative thermal stability of the a and b enzymes (Fig. 5), as well as their different stabilities in crude extracts, may explain why previous investigations of the Dictyostelium enzyme did not detect the b form.

With the four buffer systems used in determining the pH optima (citrate, imidazole, MES, and triethanolamine), the a enzyme showed a preference for MES buffer over imidazole buffer systems used in the presence of 4 mM P and P, K values in the presence of 4 mg/ml glycogen. The 5′-AMP-dependent enzyme showed P, K values of 1.5 and 55 mM (A) and glycogen K values of 4.2 and 150 mg/ml (C) with (O) and without (•) 3 mM 5′-AMP, respectively. The independent enzyme showed P, (B) and glycogen (D) K values of 1.2 mM and 2.7 mg/ml, respectively, in the absence of 5′-AMP. All data points are the mean of triplicate determinations. Lines were drawn by regression analysis and gave r values of no less than 0.9.

![Figure 7](image-url)  **FIG. 7. Determination of the 5′-AMP K for the dependent glycogen phosphorylase.** Glycogen phosphorylase activity in the presence of increasing concentrations of 5′-AMP is shown. Undiluted (C), 2-fold diluted (O) and 4-fold diluted (•) enzymes are shown. Each determination gives a 5′-AMP K of ~700 μM and an activity ratio of ~0.08. Data points represent the mean of triplicate determinations.

![Figure 8](image-url)  **FIG. 8. Phosphate (P) and glycogen K values of both forms of glycogen phosphorylase.** Glycogen K values were determined in the presence of 4 mM P and P, K values in the presence of 4 mg/ml glycogen. The 5′-AMP-dependent enzyme showed P, K values of 1.5 and 55 mM (A) and glycogen K values of 4.2 and 150 mg/ml (C) with (O) and without (•) 3 mM 5′-AMP, respectively. The independent enzyme showed P, (B) and glycogen (D) K values of 1.2 mM and 2.7 mg/ml, respectively, in the absence of 5′-AMP. All data points are the mean of triplicate determinations. Lines were drawn by regression analysis and gave r values of no less than 0.9.
Glycogen Phosphorylase in Dictyostelium

![Figure 9. Effects of changing concentrations of P, on glycogen on glycogen and P, K, values, respectively, of both forms of glycogen phosphorylase. Glycogen K, values were determined in the presence of 4 mM P, and P, K, values in the presence of 4 mg/ml glycogen. Phosphate (A) and glycogen (C) K, values of the dependent enzyme were determined in the presence of 5 mM 5'-AMP; those of the independent enzyme (B and D, respectively) were determined in the absence of 5'-AMP. The numbers in each panel indicate the concentrations of glycogen (mg/ml) or phosphate (mM) used in the determination of P, and glycogen K, values, respectively. Data points are the mean of triplicate determinations. Lines were drawn by regression analysis and gave r values of no less than 0.9.](image)

Fig. 9. Effects of changing concentrations of P, on glycogen on glycogen and P, K, values, respectively, of both forms of glycogen phosphorylase. Glycogen K, values were determined in the presence of 4 mM P, and P, K, values in the presence of 4 mg/ml glycogen. Phosphate (A) and glycogen (C) K, values of the dependent enzyme were determined in the presence of 5 mM 5'-AMP; those of the independent enzyme (B and D, respectively) were determined in the absence of 5'-AMP. The numbers in each panel indicate the concentrations of glycogen (mg/ml) or phosphate (mM) used in the determination of P, and glycogen K, values, respectively. Data points are the mean of triplicate determinations. Lines were drawn by regression analysis and gave r values of no less than 0.9.

with the preferred pH range of the phosphorylase a enzyme that becomes active at the slug stage.

Activation of the b enzyme by 5'-AMP results in a 35-fold increase in the substrate K, values (glycogen and P,) with no apparent change in V max. The a form of the enzyme requires no activation and displays increased P, and glycogen K, values, (45- and 55-fold, respectively) over those of the non-activated b form (Fig. 8). As is typical of two substrate allosteric enzymes, the kinetic constants vary with changing substrate concentrations (Fig. 9).

We have attempted to demonstrate conversion of the a to b forms by phosphorylation and dephosphorylation. For example, Dictyostelium b activity was tested as a substrate for commercial rabbit-muscle phosphorylase kinase. No conversion of the Dictyostelium enzyme to a 5'-AMP-independent form was observed, whereas parallel samples of rabbit muscle phosphorylase were completely converted to the a form. Likewise, there was no incorporation of 32 P, into the Dictyostelium b enzyme when incubated in the presence of 3-labeled ATP and rabbit muscle phosphorylase kinase. The reciprocal experiment in which rabbit muscle phosphorylase b was incubated with various Dictyostelium extracts and ATP did not result in conversion to the a activity. In addition, the Dictyostelium b activity could not be converted to an a form when crude extracts were incubated in a variety of reaction mixtures that are known to support the conversion in other systems. We also attempted to convert the a form to the b form by dephosphorylation. No conversion to a 5'-AMP-dependent form was observed when the extracts were incubated under conditions that favor protein phosphatases or when commercially available phosphatases were included in the incubation mixtures.

Previous investigators of the Dictyostelium phosphorylase were not aware of a 5'-AMP-dependent activity that is present early in development, but did observe a 5'-AMP-independent form that accumulated late in development. All three of these reports lead to the conclusion that the mechanism for the increase in activity during development was an increase in de novo synthesis of the protein. Thomas and Wright (16) used antisera to quantitate the amount of precipitable phosphorylase protein from cell extracts of different stages of development. They showed a concomitant increase of phosphorylase activity and immunoprecipitable protein during development. Firtel and Bonner (32) concluded that the phosphorylase required prior RNA and accompanying protein synthesis, on the basis of the effect of actinomycin D and cycloheximide on the accumulation of the enzyme. Higgins and Dahmus (18) used cell-free translation of poly(A)-containing RNA to show that the level of glycogen phosphorylase mRNA is undetectable until post-aggregation and is maximum late in development. Thus, the literature argues strongly that the late stage enzyme appears as a result of de novo synthesis, rather than by an epigenetic conversion from the form that we have observed early in development. The results from Fig. 10 of the peptide maps of the a and b forms support the idea that they are two different proteins. We are currently preparing antisera against the proteins, to be used in an immunological study of the two forms, as well as cloning the gene(s) for both forms. Upon completion of these studies we anticipate being able to distinguish between genetic or epigenetic regulation of the enzyme during cellular differentiation.

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