Developmental Regulation of Alkaline Phosphatase in *Dictyostelium discoideum*

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The kinetics of accumulation of alkaline phosphatase activity were determined in cells of wild-type and morphologically aberrant mutant strains of *Dictyostelium discoideum* induced to develop synchronously on membrane supports. The enzyme specific activity increased slowly in wild-type cells until culmination when a dramatic rise in specific activity occurred. The patterns of accumulation in the mutant strains, as well as previous electrophoretic analysis, suggest that the two phases of accumulation may result from the synthesis of distinct isozymes. The rapid accumulation of alkaline phosphatase was found to require concomitant protein synthesis. Ribonucleic acid synthesis, on the other hand, could be inhibited during the 8 hr immediately preceding culmination without affecting the amount of enzyme accumulated. When ribonucleic acid synthesis was inhibited earlier in development, the accumulation of alkaline phosphatase was reduced. Comparison of these results with work on other developmentally controlled enzymes suggests that both transcriptional and translational control occurs during development of *D. discoideum*. The accumulation of alkaline phosphatase was shown to require specific cellular topography during culmination, suggesting that intercellular interactions which allow synthesis of alkaline phosphatase occur at that stage.

One approach to the elucidation of the biochemical basis of development in multicellular organisms involves the description of developmentally regulated proteins. Proteins which have enzymological activity are particularily suitable for such an analysis since they can be quantitatively measured in whole-cell extracts.

Development of the cellular slime mold *Dictyostelium discoideum* can be synchronously initiated by removing the source of nutrients (14). A series of biochemical and cytological differentiations which result in fruiting body construction then proceed in the absence of net growth or cell division. Following the initiation of development, the myxamoebae aggregate to form pseudoplasmodia of approximately 10^5 cells; then, after a period of migration, the pseudoplasmodia culminate to form sorocarps consisting of about 7×10^4 spores supported by a tapering cellular stalk. During this process, the specific activity of alkaline phosphatase (EC 3.1.3.1) has been found to increase by a factor of more than five (2, 3).

To investigate the developmental regulation of this enzyme, I have studied the detailed kinetics of accumulation of activity in wild-type cells and in cells of several morphologically aberrant mutant strains. Moreover, the requirements for ribonucleic acid (RNA) and protein synthesis have been determined. The results indicate that the increase in specific activity of alkaline phosphatase requires concomitant protein synthesis and prior RNA synthesis and is an integral part of slime mold development. Some factors which control the accumulation of this protein are described.

METHODS AND MATERIALS

Chemicals. *p*-Nitrophenyl phosphate was purchased from Calbiochem, Los Angeles, Calif. Cycloheximide was purchased from The Upjohn Co., Kalamazoo, Mich. Actinomycin D was the kind gift of Merck & Co., Inc., Rahway, N.J. Tris(hydroxymethyl)-aminomethane (Tris) was purchased from the Sigma Chemical Co., St. Louis, Mo. Membrane filter supports (AABP 047) were purchased from the Millipore Corp., Bedford, Mass.

Organisms. *D. discoideum* NC-4 (haploid) was isolated and described by Raper (8). All mutant strains employed in this study were derived from this strain. Strain FR-17 carries out development at an accelerated rate and has been described by Sonneborn et al. (12). Strain KY-3 forms pseudoplasmodia but fails to culminate in fruiting bodies (18). Strain KY-19 forms spores but very little stalk (1). Strain

VA-4 fails to aggregate (5). Strains NC-4, FR-17, KY-3, and KY-19 are from the collection of M. Sussman.

Conditions for growth and development. The procedures described by Sussman (14) were followed. Amoebae were grown in association with *Aerobacter aerogenes* on a Bacto-peptone, yeast extract medium solidified with 2% agar. Development was initiated by collecting growing cells from plates, washing them free of bacteria by differential centrifugation, and depositing them on membrane supports saturated with a solution containing NaCl (9 mg/ml), streptomycin sulfate (0.5 mg/ml), and potassium phosphate buffer (10^{-3} M) at pH 6.5.

Cell extracts. At various times after the initiation of development, approximately 10^8 cells were washed off the membrane supports with 3 ml of cold distilled water. The samples were frozen. After thawing, the cells were ultrasonically disrupted with a Bronson Sonifier at a tip energy of 40 w for 30 sec. Alkaline phosphatase activity in this extract was assayed within 30 min. This procedure resulted in maximal measurable phosphatase activity in both myxamoebae and completed fruiting bodies.

Alkaline phosphatase assay. Samples of whole extracts were incubated with 10^{-2} M *p*-nitrophenyl phosphate in 10^{-2} M Tris-hydrochloride buffer (pH 8.5) containing 10^{-2} M MgCl$_2$ at 25 C. The reaction was stopped by adding an equal volume of 1 M Na$_2$CO$_3$. The change in optical density at 420 nm was determined on a Zeiss spectrophotometer. The assay is linear with respect to time for more than 1 hr. It is linear with respect to amount of extract from 10 to 100 μg of protein per ml of assay mixture. One unit of activity is defined as that amount which will liberate 1 nmole of nitrophenol per min under the above conditions. Specific activity is expressed as units per milligram of protein.

Protein estimation. The method of Lowry et al. (7) was used to estimate protein concentration. Crystalline bovine serum albumin was used as a standard.

RESULTS

Kinetics of accumulation of alkaline phosphatase. Gezelius and Wright (2) measured the specific activity of alkaline phosphatase at several morphological stages in the development of *D. discoideum* and reported an essentially constant rate of accumulation of this enzyme following the initiation of development. Greater resolution can be achieved when the cells are induced to develop synchronously on membrane supports (Fig. 1). The specific activity of alkaline phosphatase was found to increase gradually during the first 18 hr of development while the myxamoebae aggregate and form pseudoplasmodia. During the subsequent 6 hr, while the pseudoplasmodia culminate to form fruiting bodies, the specific activity was found to increase rapidly by a factor of about four. Thereafter, the specific activity decreased. It is clear that the

majority of the accumulation of alkaline phosphatase occurs only during culmination.

Requirements for protein and RNA synthesis. Protein synthesis in *D. discoideum* can be preferentially inhibited more than 85% if cycloheximide is added before culmination (1, 13). When protein synthesis was blocked either 14 or 18 hr after the initiation of development, the accumulation of alkaline phosphatase stopped abruptly (Fig. 1). The activity which had already accumulated at the time of addition of cycloheximide was stable over the subsequent 10 hr. It is concluded that the accumulation of alkaline phosphatase requires concomitant protein synthesis and that the enzyme is stable, at least in the presence of cycloheximide.

RNA synthesis in *D. discoideum* can be preferentially inhibited more than 95% by actinomycin D (16, 17). When actinomycin D was added to cells 18 hr after the initiation of development, alkaline phosphatase accumulated at the same rate as in the control cells and reached a peak specific activity essentially identical to that in the control cells (Fig. 2). Thus, the accumulation of alkaline phosphatase does not appear to require concomitant RNA synthesis.

When actinomycin D was added to aggregating cells 10 hr after the initiation of development, alkaline phosphatase accumulated to the same peak specific activity but the accumulation was delayed about 5 hr (Fig. 2). When RNA synthesis was inhibited by actinomycin D 4 hr after the initiation of development, very little alkaline phosphatase accumulated at any time (Fig. 2).

The peak specific activities of alkaline phosphatase which occurred after addition of actinomycin D at various times during development are shown in Fig. 3. The curve represents the amount of enzyme activity which can accumulate in the absence of RNA synthesis. If the accumulation of alkaline phosphatase requires synthesis of the enzyme on messenger RNA (mRNA), then this curve may represent the kinetics of accumulation of alkaline phosphatase mRNA. This mRNA appears to be stable for at least 14 hr.

Accumulation of alkaline phosphatase in morphological mutants. A large number of mutant strains which develop abnormally have been isolated (12, 15, 18). One of the most interesting strains, FR-17, carries out all of the biochemical and cytological differentiations that are known to occur in wild-type cells but does so in about two-thirds the time required for development in the wild-type strain (12). If a specific biochemical event is integrated in the sequence of differentiations which result in fruiting-body forma-

tion, then it will be found to occur precociously in strain FR-17.

The kinetics of accumulation of alkaline phosphatase in strain FR-17 are shown in Fig. 4. The specific activity rises rapidly from 12 to 16 hr after the initiation of development. The specific activity reaches a peak somewhat higher
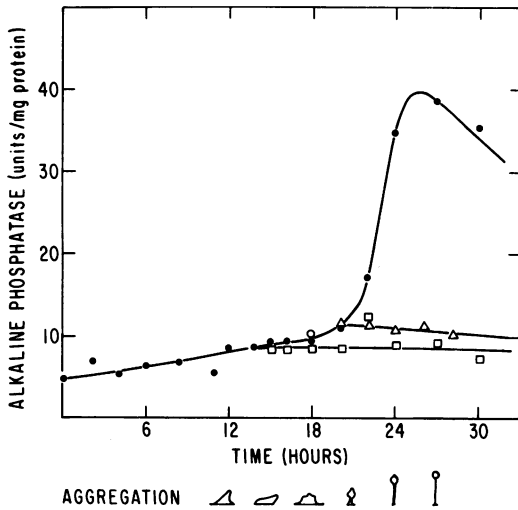

FIG. 1. *Kinetics of accumulation of alkaline phosphatase. Cells of D. discoideum were collected from membrane supports at various times after the initiation of development. The specific activity of alkaline phosphatase was determined in control cells (●) and in cells treated with cycloheximide (400 μg/ml) at 14 hr (□) and at 18 hr (△). Schematic representation of morphogenesis in control cells is presented with the time scale.*
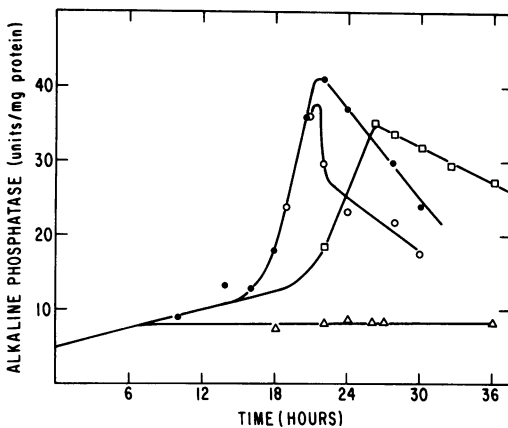

FIG. 2. *Effect of actinomycin D on accumulation of alkaline phosphatase. The specific activity of alkaline phosphatase was determined in control cells (●) and in cells treated with actinomycin D (100 μg/ml) at 4 (△), 10 (■), and 18 hr (○) after initiation of development.*
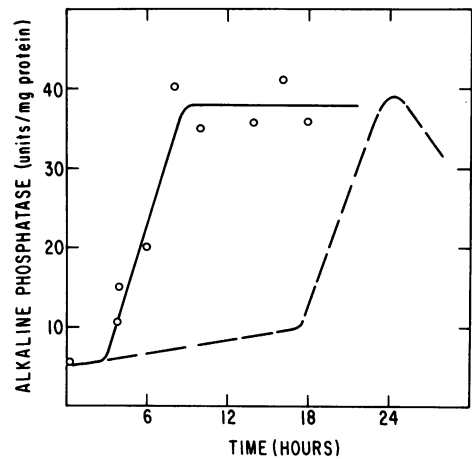

FIG. 3. *RNA synthetic period for alkaline phosphatase. The peak specific activity of alkaline phosphatase which accumulated after treatment of the cells with actinomycin D (100 μg/ml) is plotted at the time of addition of the drug. The dotted line indicates the period during which alkaline phosphatase activity accumulates.*
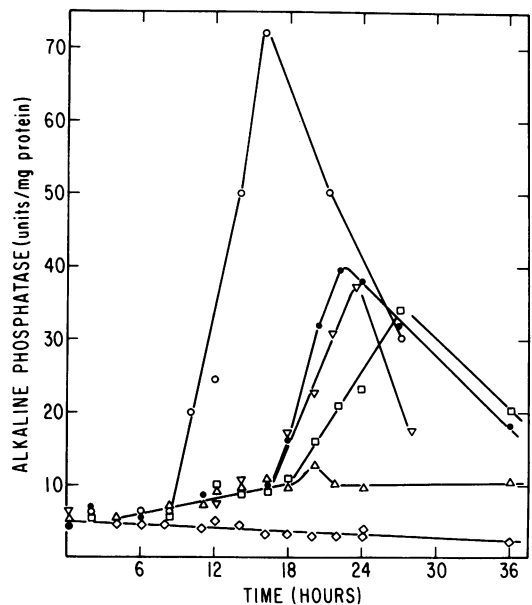

FIG. 4. *Alkaline phosphatase in morphologically aberrant mutants of D. discoideum. The specific activity of alkaline phosphatase was determined at various times after the initiation of development in strain FR-17 (○); strain NC-4 (wild-type) (●), strain min 2 (▽), strain KY-19 (□), strain KY-3 (△), and strain VA-4 (◇).*

than occurs in wild-type cells and then gradually declines. It is apparent that this enzyme accumulates precociously in the temporally deranged mutant.

Cells of strain *min 2* form morphologically normal fruiting bodies but the cells of the sorus fail to encapsulate to form spores (4). Nevertheless, alkaline phosphatase accumulates in an essentially normal manner in this strain (Fig. 4). Cells of strain KY-19 form normal spores but make very few stalk cells. Alkaline phosphatase accumulates to approximately the normal peak specific activity in this strain as well (Fig. 4). The rate of accumulation in this strain is less than in wild-type cells, resulting in a delay in the attainment of peak specific activity. It is apparent that cytodifferentiation of neither spores nor stalk cells is required for the accumulation of alkaline phosphatase.

Strain KY-3 forms pseudoplasmodia but fails to culminate. In this strain, the specific activity of alkaline phosphatase gradually doubles during the first 18 hr after initiation of development, but it then remains constant (Fig. 4).

Strain VA-4 fails to aggregate and does not accumulate alkaline phosphatase (Fig. 4). In fact, the specific activity slowly declines in cells of this strain during incubation on the membrane supports.

It appears that the initial slow rise in specific activity of alkaline phosphatase occurs in strains able to aggregate and the later rapid increase in specific activity occurs only in strains which are able to culminate.

Inhibition of accumulation. Culminating pseudoplasmodia can be dissociated to small clumps of cells by collecting in cold distilled water and triturating for 30 sec (6). When the cells are redeposited on membranes, they reaggregate and form small pseudoplasmodia within 2 hr. Fruiting body construction is delayed less than 2 hr by this procedure. Dissociation does not affect viability of the cells or the rate of incorporation of ^{14}C-amino acids in protein (6). However, accumulation of alkaline phosphatase is strongly inhibited by dissociation of culminating pseudoplasmodia at 18 hr (Fig. 5).

The process of dissociation per se does not seem to account for the inhibition of accumulation since cells dissociated 14 hr after the initiation of development accumulated alkaline phosphatase to the normal extent (Fig. 5). It appears that specific cellular topography is required at the time of culmination for the normal accumulation of alkaline phosphatase.

DISCUSSION

During the development of *D. discoideum*, alkaline phosphatase accumulates at a low rate during aggregation and pseudoplasmodium formation and then accumulates rapidly during
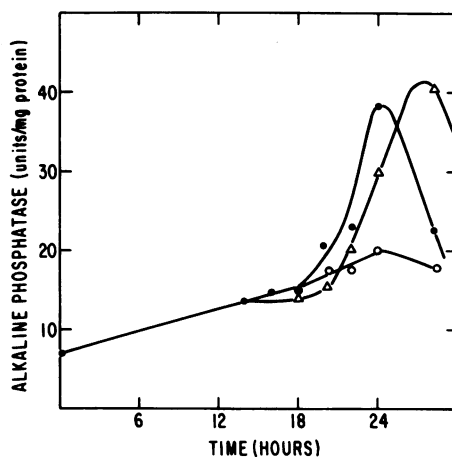

FIG. 5. *Effect of cellular topography on accumulation of alkaline phosphatase. The specific activity of alkaline phosphatase was determined in cells allowed to develop normally (●) and in cells dissociated from pseudoplasmodia at 14 hr (△) and at 18 hr (○).*

culmination. It seems quite possible that different proteins, each of which has alkaline phosphatase activity, are responsible for the two stages in accumulation. Evidence in support of this view has been provided by Solomon, Johnson, and Gregg (11), who have shown that developing cells of *D. discoideum* contain two electrophoretically separable forms of alkaline phosphatase. One form is present throughout development. The other can be observed only during culmination.

The patterns of accumulation of alkaline phosphatase in morphological mutants are consistent with the occurrence of two isozymes of alkaline phosphatase. Thus, the kinetics of accumulation in the wild-type strain and in strains FR-17, *min 2*, and KY-19 suggest that both forms are synthesized in these strains; the kinetics of accumulation in strain KY-3 suggest that the first isozyme but not the second is synthesized in this strain. The aggregateless strain, VA-4, does not appear to synthesize either enzyme following removal of the food source.

Since greater accuracy is possible in the study of the more rapidly accumulating enzyme, further developmental analysis has been restricted to the alkaline phosphatase which accumulates during culmination.

The increase in specific activity of alkaline phosphatase requires concomitant protein synthesis (Fig. 1). Previously accumulated enzyme is stable in the presence of cycloheximide. If the enzyme is stable in cells able to synthesize protein, then we can conclude that the increase in

specific activity results from an increase in the rate of de novo synthesis of the enzyme.

The increase in specific activity of alkaline phosphatase does not require the synthesis of new RNA molecules during a period of 8 hr prior to the period of accumulation (Fig. 2 and 3). However, RNA synthesis during the first 10 hr of development is required for alkaline phosphatase to accumulate. If we assume that translation of mRNA into active alkaline phosphatase occurs during culmination, then we can conclude that this mRNA is stable for at least 8 hr. The increase in enzyme-forming capacity from 4 to 10 hr of development may indicate the period of synthesis of alkaline phosphatase mRNA.

These results can be compared with those found in similar studies of other developmentally controlled enzymes of *D. discoideum* (Fig. 6). It can be seen that there are significant differences between the periods of RNA synthesis required for specific enzymes and the periods of accumulation of the various enzymes. Thus, both an early enzyme, acetylglucosaminase (EC 3.2.1.30), and a late one, β-glucosidase (EC 3.2.1.21), accumulate within 4 hr of the RNA synthetic period, yet alkaline phosphatase does not accumulate for 14 hr after the RNA synthetic period. Differential control at the level of translation most easily accounts for these observations.

The accumulation of alkaline phosphatase appears to require a specific morphological arrangement of the cells during the period of culmination, since dissociation of cells 18 hr after the initiation of development inhibits subsequent accumulation of alkaline phosphatase at least 75% (Fig. 5). The accumulation of another developmentally controlled enzyme, uridine diphospho (UDP)-galactose polysaccharide transferase, as well as total protein synthesis, is unaffected by dissociation during this period (6). Thus, it appears that certain biochemical differentiations are dependent on processes of morphogenesis while others are essentially independent.

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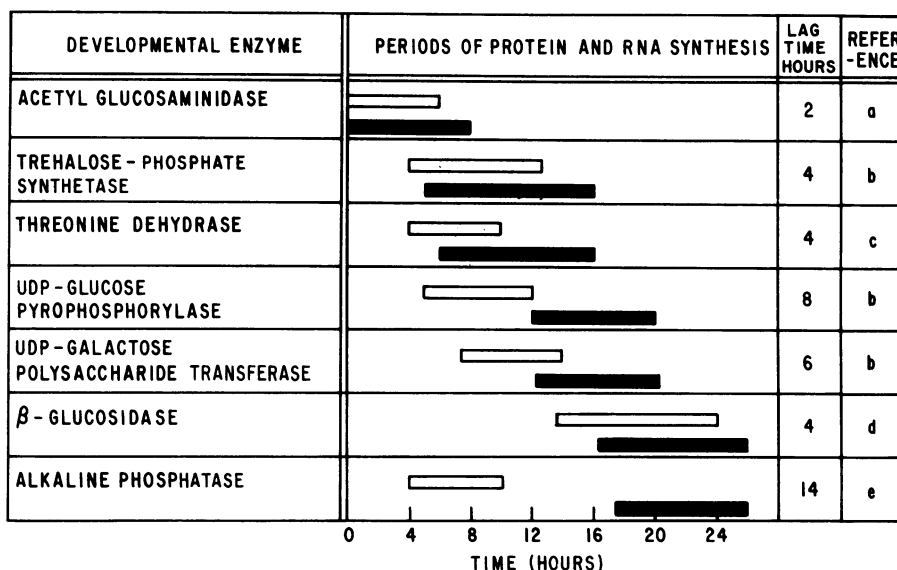| DEVELOPMENTAL ENZYME	PERIODS OF PROTEIN AND RNA SYNTHESIS	LAG TIME HOURS	REFER-ENCE
ACETYL GLUCOSAMINIDASE		2	a
TREHALOSE - PHOSPHATE SYNTHETASE		4	b
THREONINE DEHYDRASE		4	c
UDP - GLUCOSE PYROPHOSPHORYLASE		8	b
UDP - GALACTOSE POLYSACCHARIDE TRANSFERASE		6	b
β - GLUCOSIDASE		4	d
ALKALINE PHOSPHATASE		14	e

0 4 8 12 16 20 24
TIME (HOURS)

FIG. 6. *Transcription and translation in development of D. discoideum. A schematic summary of the periods of RNA synthesis (open bars) and protein synthesis (filled bars) required for accumulation of seven developmentally controlled enzymes: (a) Loomis (5); (b) Roth et al. (10); (c) Allister and Loomis, unpublished data; (d) Coston and Loomis, in preparation; (e) Fig. 1–3. Lag times were measured from the midpoint of the RNA synthetic period to the midpoint of the protein synthetic period for each developmentally controlled enzyme.*

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