Glycogen Phosphorylase in Dictyostelium discoideum

II. SYNTHESIS AND DEGRADATION DURING DIFFERENTIATION*

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A purified preparation of glycogen phosphorylase from Dictyostelium discoideum was used to elicit specific antisera in rabbits. The antisera were used to quantitate the amount of precipitable phosphorylase protein from cell extracts prepared at various stages of the developmental cycle. Following isotope incorporation studies in differentiating cells, the specific radioactivity of enzyme isolated by antibody precipitation was compared to that of acid-insoluble protein. Prior to 5 hours of development, glycogen phosphorylase could not be detected enzymatically or immunologically. Between aggregation and culmination, the rate of enzyme synthesis increased about 6-fold, then decreased to an insignificant value in young sorocarps. The rate of enzyme degradation was negligible during the period of maximal enzyme accumulation, then increased to a peak value of 40% after culmination, coincident with a rapid drop in phosphorylase activity. The data indicated that the increase in glycogen phosphorylase activity during development results from an increase in the rate of enzyme synthesis.

Changes in the activity of glycogen phosphorylase during differentiation in Dictyostelium discoideum have been observed previously (1, 2). In the latter study, experiments with inhibitors and developmental mutants led the authors to suggest that changes in phosphorylase activity required prior RNA and concomitant protein synthesis. Thus, they concluded that the appearance of glycogen phosphorylase was a developmentally controlled enzyme dependent upon specific periods of transcription and translation. However, as recently demonstrated in the cases of uridine diphosphate (UDP)-glucose pyrophosphorylase (3), trehalose-6-phosphate synthetase (4), and two glycosidases (5, 6), mechanisms other than increased rate of enzyme synthesis are inhibited by actinomycin D and cycloheximide. For these and many other reasons (see review (7)), the use of inhibitors in this system is not a sufficient basis for obtaining evidence of gene activation. To obtain such evidence, it must be shown that an increase in enzyme protein occurs due to an increase in the rate of its synthesis.

The present investigation is concerned with whether the changes in glycogen phosphorylase activity which occur during development correspond to changing quantities of enzyme protein. If so, the next question of interest is whether these changes are due to variations in the rate of enzyme synthesis, degradation, or both. In order to investigate changes of phosphorylase activity in vivo, the enzyme was purified to homogeneity and antibody prepared against the purified enzyme. The antibody was used to precipitate phosphorylase protein from extracts prepared at various stages of development. The rate of enzyme synthesis was evaluated by measuring the extent of [35S]methionine incorporated in vivo into enzyme protein relative to isotope incorporated into total acid-insoluble (AI) protein. In "chase" experiments, protein turnover was estimated from the rate of loss of radioactivity in enzyme and in AI protein.

EXPERIMENTAL PROCEDURE

Chemicals—Freund's complete adjuvant and prepared immunodiffusion dishes were obtained from Cappel Laboratories. Acrylamide gel reagents were purchased from Canalco Co. Sodium lauryl sulphate was obtained from Sigma Chemical Co. and [3H]methylamine (specific activity 235 Ci/mmol) and Aquasol from New England Nuclear Corp. Reagents used for the assay and purification of glycogen phosphorylase are listed in a previous paper (8).

Assay—The assay of glycogen phosphorylase and the definition for a unit of enzyme activity are described in a previous paper (8). Protein was determined by the method of Lowry et al. (9).

Enzyme Purification—Glycogen phosphorylase was purified from Dictyostelium discoideum cells at the culmination stage of development, as described in the preceding paper (8). Approximately the same overall increase in specific activity was obtained and the ratio of each increase during individual steps was similar.

Immunodiffusion Analysis—Ouchterlony double immunodiffusion analysis in prepared dishes was performed as outlined by Ouchterlony (10). The dishes containing enzyme sample and antisera were incubated at room temperature (25°) for 12 to 15 hours. Precipitin bands formed between antibody and enzyme protein were stained with aniline blue black (5 mg/ml in 7% acetic acid).

Preparation of Phosphorylase Antisera—The purified phosphorylase...
(2 mg) in Freund's complete adjuvant was injected into multiple sites into a rabbit according to the procedure of Bernier and Cebras (11). The animal was bled (50 ml) at weekly intervals beginning after the 2nd week of antigen challenge. Antiserum was prepared by fractionation of the rabbit serum with ammonium sulfate. The protein precipitating between 0 to 50% salt saturation was collected by centrifugation. The precipitate was dissolved and dialyzed overnight at 6°C against 0.9% NaCl in 10 mM sodium phosphate, pH 7.0. Control antisera obtained from rabbits receiving injections of Freund's adjuvant only were treated in a similar manner. The fractionated antisera were stored at -20°C.

Isotope Incorporation—Vegetative amoebae were harvested, washed free of bacteria, and aliquots equivalent to approximately 0.9-ml packed cell volumes were spread evenly on a 12.5-cm diameter Whatman No. 50 filter paper disc. The filter paper was suspended in a 5% agar layer containing 10 mM phosphate buffer, pH 7.2, in a final volume of 0.125 ml. The mixture was incubated at 25°C for 1.0 hour. After centrifugation at 20,000 x g for 30 min, the pellet was washed three times with 2.0 ml of 0.9% NaCl.

Isolation of Enzyme and Acid-insoluble Protein from Labeled Cells—Labeled cells prepared as described above were thawed at room temperature and centrifuged (4°C) at 27,000 x g for 15 min. The crude supernatant was treated with streptomycin sulfate to a final concentration of 2% w/v. The streptomycin sulfate fraction was washed at 6°C for 1.0 hour, then centrifuged at 27,000 x g for 20 min. The supernatant was then treated at 30°C for 30 min to precipitate any extraneous material prior to enzyme-antiserum incubation. This procedure did not affect phosphorylase activity. After centrifuging the heated extract at 27,000 x g for 20 min, the resulting supernatant was separated into two aliquots. Aliquot A, aliquots of prepared extracts (0.5 ml) were treated with 5% trichloroacetic acid for the determination of specific radioactivity of total acid-insoluble protein (13). The trichloroacetic acid-precipitated protein was boiled for 10 min and pelleted by centrifugation at 1,200 x g for 10 min. The pellet was washed three times in 10% trichloroacetic acid and the protein resuspended in 0.5 ml of 0.1 N NaOH. These samples along with an equal volume of 0.2% acetic acid were added to 10 ml of Aquasol and counted for [35S]methionine in a Beckman liquid scintillation spectrometer. Aliquot B, aliquots of prepared extracts (1.6 ml) were incubated in conical centrifuge tubes with an excess of antisera for 1.0 hour at room temperature (23°C). The enzyme-antibody complex was pelleted by centrifugation at 20,000 x g for 30 min. The pellet was washed twice with 2.0 ml of 0.9% NaCl.

Enzyme Activity Assay—The washed enzyme-antibody complex was suspended in 50 μl of 10 mM sodium phosphate buffer, pH 7.0, containing 1.0% Na dodecyl-SO4, 10% 2-mercaptoethanol and incubated overnight at 37°C. The dissolved pellets were subjected to electrophoresis in 6% acrylamide gels according to the procedure of Weber and Osborn (14). Electrophoresis was carried out at room temperature using a constant current of 5 mA/gel. The gels were stained overnight in 50% methanol/10% acetic acid solution containing 2.4 g of fast green dye/100 ml of solution. The gels were destained electrophoretically in 100% methanol/10% acetic acid and then allowed to swell overnight in 7.5% acetic acid.

Determination of Phosphorylase-specific Radioactivity—The de-stained Na dodecyl-SO4 gels were scanned with a Joyce-Loebel microdensitometer equipped with a 586-nm filter. The phosphorylase protein band was determined from the densitometer scan by comparison with a control scan of purified slime mold phosphorylase protein.

Quantitation of stained enzyme protein was determined according to the procedure outlined by Gustafson et al. (33). After recording the staining pattern, the gels were cut into 3.0-mm sections. Each slice in a scintillation vial was hydrolyzed with 0.5 ml of 30% H2O2, at 70°C for 1.5 hours. After cooling and addition of 10 ml of Aquasol, the vials were counted for 10 min in a Beckman liquid scintillation spectrometer.

Results

Enzyme Titrations—A specific phosphorylase antiserum was prepared by injecting the purified enzyme into a rabbit. An Ouchterlony double diffusion analysis of the prepared antisera indicated a single precipitin line when reacted against the purified phosphorylase. Normal rabbit sera were tested as a control and failed to show a precipitin band. Titration of the antiserum with purified enzyme is shown in Fig. 1. In the region of antibody excess, no enzyme activity remained in the supernatant after centrifugation of the titration mixture. Antiserata-treated crude extracts were also devoid of enzyme activity. The enzyme-antibody complex isolated under these conditions was completely inactive. Titration of phosphorylase in the presence of glycogen, phosphate, or 5‘ AMP failed to prevent enzyme precipitation. It can be calculated from Fig. 1 that 1.0 ml of antisera will precipitate (neutralize) 6.5 μg units of the slime mold phosphorylase. A similar result was obtained when the purified enzyme was titrated with antisera.

Developmental Profile—The activity of glycogen phosphorylase was determined throughout the developmental period of Dictyostelium discoideum (Fig. 2). Enzyme activity first appears approximately 5 to 6 hours after the beginning of starvation and continues to increase markedly during the aggregation and pseudoplasmodium stages. Phosphorylase activity reaches a maximum level at culmination (18 hours) and then decreases during sorocarp formation (20 to 24 hours). It has been previously shown that the rise and fall of enzyme activity is not due to the presence of soluble activator or inhibitor molecules in crude extracts (1, 2). Preparation of extracts in the presence of substrates, 5‘-AMP, or glycero did not alter the amount of enzyme activity recovered at any stage of development. In addition, the slime mold enzyme did not exhibit the cold-sensitive property of the phosphorylase studied from rabbit muscle (15). Thus, no evidence has been observed which would indicate differential enzyme stability.

As the activity of the slime mold phosphorylase undergoes

![Fig. 1. Titration of antisemum with purified glycogen phosphorylase.](http://www.jbc.org/)
somewhat dramatic shifts during development, rabbit antisera were used to measure the amount of enzyme protein present at various stages of the developmental cycle. Prepared streptomycin sulfate-treated crude extracts were precipitated with excess antisera and subjected to Na dodecyl-SO₄ gel electrophoresis. After staining, the gels were quantitated for phosphorylase protein by scanning with a microdensitometer. Quantitatively, the amount of antigenic protein parallels the rise and fall of enzyme activity between late aggregation (10 hours) and sorocarp formation (24 hours) (Fig. 2). Prior to aggregation (10 hours), the sensitivity of the assay is such that it is not possible to determine whether the protein present in the enzyme region is catalytically active. However, an attempt was made to purify amoebae extracts (4-hour cells), using the same procedure as outlined for extracts from culminating cells (8), and the preparation was then subjected to Ouchterlony analysis and gel electrophoresis. No precipitin band was evident, nor was protein present in the enzyme region on the gel (see previous paper). Between aggregation and culmination the enzyme accumulates from 0.025% of the total cell protein to 0.11%. During sorocarp formation (24 hours), enzyme protein drops to about 0.07% of the total protein. It should be noted that after aggregation, the relationship between antibody-precipitable protein and enzyme activity argues against the possibility that phosphorylase increases in activity as a result of a modification of pre-existing enzyme molecules. However, this situation cannot be completely ruled out, as there might exist a catalytically inactive precursor which is not immunologically reactive.

Measurement of Isotope Incorporation into Phosphorylase Protein—To determine the fraction of radioactivity actually incorporated into glycogen phosphorylase protein, antisera precipitates prepared from cells exposed to [35S]methionine were redisolved in Na dodecyl-SO₄, 2-mercaptoethanol, and treated electrophoretically on 6% Na dodecyl-SO₄ acrylamide gels. After electrophoresis, the gels were stained, quantitated for protein, and sectioned for counting as outlined under “Experimental Procedure.” Fig. 3 represents the results from one such experiment in which enzyme protein was precipitated from cell extracts labeled for 3 hours. A comparison of the electrophoretic pattern of precipitated slime mold enzyme (lower gel) with purified rabbit muscle phosphorylase (upper gel) shows that the main radioactive band coincides with the subunit band of the rabbit muscle enzyme. The subunit molecular weight of the purified slime mold phosphorylase has previously been determined to be the same as the rabbit muscle enzyme (95,000) (8). When prepared extracts were treated with control serum and analyzed electrophoretically, only a low level of background radioactivity was observed. The major protein band appearing in Na dodecyl-SO₄-treated precipitates was heavy chain γ-globulin which could be completely separated from the enzyme subunit during electrophoresis. In addition, the lower gel also showed some minor protein components. However, all of these minor contaminant bands were well separated from the enzyme protein and none appeared to be associated with significant radioactivity. Evaluation of total counts incorporated into enzyme protein were corrected for background interference by subtracting the counts per min of the gel section preceding those containing the enzyme band. Results are expressed in terms of specific radioactivity (counts per min per mg of enzyme protein) incorporated into enzyme protein to minimize the error associated with incomplete antigen recovery.

A control experiment was done to ascertain the variability of the procedure used to determine label incorporation into enzyme and AL protein. Three separate cell lots were pulse-labeled with [35S]methionine (3 hours) over the same period of development (14 to 17 hours). Each of the cell lots was
evaluated independently for radioactivity incorporated into enzyme and AI protein. Calculations revealed that the variation in specific radioactivity of enzyme or AI protein was no greater than 15%.

**Isotope Incorporation into Enzyme and Acid-insoluble Protein during Development**—In order to investigate the *in vivo* regulation of phosphorylase activity during development, pulse- and pulse-chase-labeling procedures were used to estimate the relative rates of enzyme synthesis and degradation. Under the labeling conditions used (see “Experimental Procedure”), cells continue to develop and accumulate glycogen phosphorylase normally over at least a 6-hour period. Figs. 4 and 5 show the results of isotope incorporation experiments performed when enzyme units per cell were increasing at a maximal rate. During this period of development (10 to 16 hours), linear rates of label incorporation into both enzyme and AI protein were observed. Based on specific radioactivity, incorporation of label into enzyme protein is about 3 times faster than that incorporated into AI protein. For example, in Fig. 5 enzyme and AI protein were labeled at rates of 11,500 and 3,950 cpm/mg/hour, respectively. While enzyme is accumulating at a rate of 0.074 unit/cell aliquot/hour, the concentration of AI protein does not change significantly over this period of time. In previous studies, the rate of AI protein synthesis and degradation (i.e. turnover) was calculated based on the specific radioactivity of endogenous [35S]methionine (Table I). This was essential because of 10-fold changes in permeability which occur during development (13). Due to permeability barriers, it is not possible to do meaningful chase experiments at the earlier stages of differentiation (19).

However, chase experiments can be carried out during culmination, when permeability is greatest. Knowing the relationship between enzyme units and micrograms of enzyme protein (Fig. 2; 1 µg of enzyme = 0.028 unit), it has been possible to estimate the rates of enzyme synthesis and degradation at various stages of development.

**Estimates of Rates of Enzyme Synthesis and Degradation**—In the case of UDP-glucose pyrophosphorylase (3, 16) and glycosidases in *D. discoideum* (5, 6), enzyme is not degraded during the period of its maximal rate of accumulation. Calculations indicate that this is also true during the period of maximal accumulation of glycogen phosphorylase (between the 10th and 16th hour of differentiation; Fig. 2). In an experiment carried out between hours 10 to 14 (Fig. 4), the rate of increase in enzyme specific radioactivity (counts per min per mg per hour) is 3-fold that of AI protein. At this stage of differentiation, the rate of average protein turnover is about 10%/hour (Table I). Thus, assuming no degradation, phosphorylase is being synthesized at 30%/hour. In fact, the increase in enzyme protein (1.3 to 3.7 µg or 0.6 µg/hour) over the 4 hours of the experiment can be almost exactly accounted for by an initial rate of synthesis of 30%/hour. The same is true for the experiment at prepulmination (Fig. 5). In this case the rate of turnover of AI protein was taken to be 8.3 (the average of 9.7 and 6.9; see Table I). Assuming no degradation, enzyme is synthesized 2.9 times faster than AI protein or an initial rate of 24%/hour. Again this rate fully accounts for the increase in enzyme from 5.6 to 8.2 µg or 1.3 µg/hour.

Chase experiments were carried out during culmination, as indicated in Fig. 6. Cells were incubated with label from the 12th to 15th hour of development, and then transferred to cold methionine and chased through hour 18. Calculations from the half-life of AI protein gives a value of about 11%/hour turnover, in rough confirmation of the earlier studies in which turnover was calculated based on the specific radioactivity of endogenous methionine (Table I). As this correspondence indicates that the chase was effective in this experiment, the loss in total counts should indicate the extent of enzyme degradation. A degradation rate based on initial total counts in enzyme gave a value of about 12%/hour. As net enzyme accumulation (synthesis minus degradation) is occurring at a

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1 G. L. Gustafson and B. Wright, unpublished experiments.
TABLE I

| Stage                  | Time  | Rate of synthesis | Rate of degradation | Net accumulation of enzyme activity |
|-----------------------|-------|------------------|---------------------|-------------------------------------|
| Amoebae-aggregation   | 4-8 h | -0.3 %/hr        | +0.3 %/hr           | -1.6 %/hr                           |
| Aggregation-pseudoplasmodium | 10-14 h | 0.6 %/hr    | +0.6 %/hr           | +10 %/hr                            |
| Preculmination        | 14-16 h | 1.3 %/hr    | 0                   | +1.3 %/hr                           |
| Culmination           | 15-18 h | 1.8 %/hr    | 12 %/hr             | +1.0 %/hr                           |
| Postculmination       | 21-23 h | 0.6 %/hr    | 40 %/hr             | -1.4 %/hr                           |
| Young sorocarp        | 22-26 h | 0.0 %/hr    | 13 %/hr             | -1.8 %/hr                           |

*Values taken from Wright and Anderson (13).

Fig. 6. Chase of [35S]methionine from prelabeled phosphorylase and acid-insoluble protein during culmination (15 to 18 hours). Cells on filter paper discs were incubated with [35S]methionine from the 12th to the 15th hour of development. After the 15th hour, the cells were transferred to cold methionine and chased through hour 18. At the designated time intervals cell extracts were prepared as outlined under “Experimental Procedure.” Results are expressed as specific radioactivity (counts per min per mg of protein) in phosphorylase protein (O-O) and acid-insoluble protein (•-•). The change in phosphorylase units per filter paper disc (A-A) over the chase period is also represented.

rate of 1.0 μg/hour, the addition of 12%/hour of the initial amount of enzyme present (6.9 μg) to compensate for enzyme degradation, gives a rate of enzyme synthesis of about 1.8 μg/hour.

Following culmination, when the enzyme level decreases, a chase (from hours 21 to 23) and incorporation (from hours 22 to 26) experiment were done. During the chase experiment enzyme decreased from 4.3 to 1.5 μg in 2 hours, or 18%/hour. A degradation rate based on total counts in enzyme gave a value of 40%/hour, necessitating a rate of enzyme synthesis of 22%/hour or roughly (using an average of 3 μg of enzyme present) 0.6 μg/hour. The fact that some synthesis must occur was also indicated by a decrease in enzyme specific radioactivity. During the incorporation experiment, from hours 22 to 26 (Fig. 7) enzyme-specific radioactivity did not change, and the amount of enzyme fell from 3.7 to 1.9 μg in 4 hours or 13%/hour. Thus, it appears that insignificant enzyme synthesis occurred. The estimates of enzyme synthesis and degradation are summarized in Table I. The value for hours 4 to 8 (amoebae-aggregation) is based on small amounts of enzyme protein and hence represent a very rough estimate.

**DISCUSSION**

Over the past 10 years, a great deal of indirect evidence has been offered in support of the hypothesis that gene activation is the critical variable which triggers enzyme accumulation (and hence differentiation) in Dictyostelium (7, 17). This hypothesis has now been examined in four cases in which a correspondence exists between enzyme activity and the amount of enzyme protein: UDP-glucose pyrophosphorylase (3, 7), β-N-acetylglucosaminidase, α-glucosidase (5, 6), and glycogen phosphorylase. In the first three cases, the authors have concluded that the rates of synthesis of these enzymes do not increase going from the growth to the differentiation phase of the life cycle. They further concluded that the increases in enzyme specific activity observed do not correspond to any change in the rate of enzyme synthesis and therefore do not implicate gene activation.

In the case of glycogen phosphorylase, gene activation could be the critical variable limiting the rate of enzyme accumulation. In contrast to the enzymes cited above, glycogen phosphorylase cannot be detected by enzymatic assay nor by immunological precipitation during the first 5 hours of starvation (8). Thus, the initiation of enzyme synthesis at 5 hours could result from the appearance of a specific mRNA or other component upon which protein synthesis depends. The rate of enzyme synthesis (micrograms per hour per cell aliquot) increases, from the time it can first be measured, about 6-fold, peaking at
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culmination and then dropping to an insignificant level in young sorocarps. It is apparent from Table I that striking changes in the rate of both enzyme synthesis and degradation contribute to alterations in enzyme level. Furthermore, these changes appear to involve more complex mechanisms than the less striking changes in the level of UDP-glucose pyrophosphorylase during differentiation (3). In mammalian systems similar observations have been made regarding the specificity and mechanisms involved in protein turnover (18).

Over the course of starvation and differentiation in Dictyostelium, the amount of AI protein and its rate of turnover decrease, cellular permeability increases to a maximum at culmination and then decreases, and the amount of glycogen phosphorylase per cell also increases and then decreases. In view of these variables, it is important to have an “internal standard” as a guide in estimating the rate of enzyme synthesis and degradation. The incorporation of isotope into AI protein is ideal, as earlier calculations of protein turnover had taken into account changes in cellular permeability (13). In such a complex system, the rate of protein synthesis must be based on the specific radioactivity of endogenous precursor at each stage of differentiation. Calculations based on the half-life of a protein following a chase are valid only if independent evidence indicates that the chase is complete (as in the experiment at culmination summarized in Fig. 6).

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REFERENCES
1. Jones, T. D. H., and Wright, B. E. (1970) J. Bacteriol. 104, 754-761
2. Firtel, R. A., and Bonner, J. (1972) Dev. Biol. 29, 85-103
3. Gustafson, G. L., Kong, W. Y., and Wright, B. E. (1973) J. Biol. Chem. 248, 5188-5196
4. Killick, K. A., and Wright, B. E. (1972) J. Biol. Chem. 247, 2967-2969
5. Every, D., and Ashworth, J. M. (1975) Biochem. J. 148, 161-167
6. Every, D., and Ashworth, J. M. (1975) Biochem. J. 146, 169-177
7. Gustafson, G. L., and Wright, B. E. (1972) CRC Crit. Rev. Microbiol. 1, 453-478
8. Thomas, D. A., and Wright, B. E. (1976) J. Biol. Chem. 251, 1253-1257
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
10. Ouchterlony, O. (1958) Prog. Allergy 5, 1-78
11. Bernier, G. M., and Cebra, J. J. (1965) J. Immunol. 95, 246-253
12. Newell, P. C., and Sussman, M. (1969) J. Biol. Chem. 244, 2990-2995
13. Wright, B. E., and Anderson, M. L. (1969) Biochem. Biophys. Acta 43, 67-78
14. Weber, K., and Osborn, M. (1968) J. Biol. Chem. 244, 4406-4412
15. Graves, D. J., Sealock, R. W., and Wang, J. H. (1965) Biochemistry 4, 296-296
16. Franke, J., and Sussman, M. (1973) J. Mol. Biol. 81, 173-185
17. Killick, K. A., and Wright, B. E. (1974) Annu. Rev. Microbiol. 28, 139-166
18. Schimke, R. T., and Katunuma, N., eds (1975) Intracellular Protein Turnover, Academic Press, New York
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