Primer Dependency of Glycogen Synthetase during Differentiation in *Dictyostelium discoideum*†

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ABSTRACT: The cellular slime mold, *Dictyostelium discoideum*, has a life cycle in which the limited number of cell types and easily recognizable stages of development offer a unique model to relate biochemical events to differentiation. Ultramicrochemical techniques were employed to assay enzyme activity and product levels in cell samples as small as 0.02 μg of dry weight in reaction volumes of 0.1 μg. The techniques utilized an amplification procedure employing the enzymatic cycling of pyridine nucleotides. Glycogen synthetase (glucose 6-phosphate independent form) was assayed in individual organisms over the time course of development. From aggregation to culmination, activity decreased and was dependent on soluble glycogen primer. From culmination to sorocarp stage, enzyme activity was independent of soluble glycogen primer. Further, the enzyme and its glycogen product were recovered in a low-spin (2000*g*) pellet fraction from sorocarp homogenates. The change in primer requirements and solubility of enzyme and product occurred during culmination. Localization studies in developing spore cells revealed trends in enzyme activities and solubilities of enzyme and product similar to those in whole organisms. Possible models of cell-specific biochemical events in *D. discoideum* are discussed.

In *Dictyostelium discoideum* two of the enzymes involved in maintaining glycogen levels, glycogen synthetase (EC 2.4.1.11) and glycogen phosphorylase (EC 2.4.1.1), play key roles in the differentiation process. The degradation of glycogen at the culmination stage of development provides the major source of precursor units for production of the end products, cellulose (Hames and Ashworth, 1974a; Rosness and Wright, 1974), trehalose (Clegg and Filosa, 1961; Ceccarini, 1967; Sargent and Wright, 1971; Hames and Ashworth, 1974a; Rosness and Wright, 1974), and acid mucopolysaccharides (White and Sussman, 1963), which accumulate during the terminal stages of differentiation. Marshall et al. (1970) have shown a threefold increase in the rate of glycogen turnover from aggregation to culmination. The rate of glycogen turnover and the rate of glycogen synthesis in these two stages of development were comparable to the rate of UDPG synthesis in the same two stages as determined by Pannbacker (1967). The close agreement of these values is evidence that UDPG is the precursor of glycogen in the intact cell and that the synthesis of glycogen is the major fate of UDPG until the culmination stage of development.

The degradative enzyme, glycogen phosphorylase, is known to increase during development, with a peak of activity coinciding with the drop in glycogen level at culmination (Jones and Wright, 1970; Firtel and Bonner, 1972). Glycogen synthetase activity, on the other hand, shows no consistent change during the differentiation cycle (Wright and Dahlberg, 1967; Weeks and Ashworth, 1972). Because the enzyme is active at all stages of development, glycogen synthetase may be regulated by substrate availability or effector levels. In this regard, Rosness et al. (1971) found a glucose 6-phosphate (G-6-P)[1] dependent (D) form and a G-6-P independent (I) form of the enzyme. The I activity decreased from the myxamoeba to the culmination stage of development, whereas the D activity increased during the same period. Thus, Wright (1966) emphasized substrate and effector control of this enzyme activity. Weeks and Ashworth (1972) have concluded that the rate-limiting factors controlling glycogen synthetase in the amoebae are the levels of the substrate UDPG and the effectors, glucose 6-phosphate and ATP.

Another level of control of glycogen synthetase in *D. discoideum* is known to exist in the conversion of the enzyme from a soluble to a particulate form (Wright et al., 1968). The particulate enzyme utilized UDPG as substrate to produce insoluble glycogen. Furthermore, the activity of the particulate enzyme is not dependent on glycogen as a primer. In the present paper, we provide additional support for the change in primer dependence of G-6-P independent glycogen synthetase, and for the insoluble nature of the enzyme and product. By utilizing microtechniques, we present data that show the precise timing of the change in primer dependency during differentiation, as well as the localization of the enzyme in prespore cells.

Experimental Procedure

Chemicals. All reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.), except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was purchased from Boehringer-Mannheim Corp. (New York, N.Y.). Agar, yeast extract, and peptone were obtained from Difco Laboratories (Detroit, Mich.). The *Neurospora* trehalase was a gift from Dr. Alfred S. Sussman (University of Michigan).

Growth and Harvesting Conditions. The amoebae of *D. discoideum* were grown in the presence of *Escherichia coli* on nutrient agar surfaces, as previously described by Liddel and Wright (1961). The amoebae were quickly washed off the surface of the agar with cold, glass-distilled water and spun in a refrigerated centrifuge (Sorvall SS-3 Automatic) at 3000 rpm. The supernatant was discarded, and the amoebae were resuspended by stirring in cold glass-distilled water. The washing procedure was repeated twice more with centrifugation at 2500 rpm.

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[1] Abbreviations used are: G-6-P, glucose 6-phosphate; ATP, adenosine 5′-triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EDTA, (ethylenedinitrilo)tetraacetic acid; UDP, uridine 5′-diphosphate; PEP, phosphoenolpyruvate; NAD, nicotinamide adenine dinucleotide; PK, pyruvate kinase; LDH, lactate dehydrogenase; NADH, reduced NAD; GDH, glutamine dehydrogenase; HK, hexokinase; P_i, inorganic phosphate.

Disks of Whatman No. 50 filter paper, layered on nonnutrient agar in petri plates (140-mm diameter) were spread with 1.3 ml of the amoebae suspension. The nonnutrient agar contained 20 g of agar and 1.0 mM EDTA in 1.0 l. of 0.01 M potassium phosphate buffer, pH 6.5. The petri plates were covered and maintained at 23 °C. At the desired stage of development, the filter paper was removed and placed on dry ice, which froze the tissue within 1 s.

The filter paper was placed in a Freeze Dry Apparatus (Model 10-800, Virtis Research Equipment, Gardiner, N.Y.), for 36 h, at −40 °C. After lyophilization, the filter papers were cut into 2 × 4 cm pieces. Each piece was stored in a lyophilization flask (screw cap vial No. 10-159-10, Virtis Co., Gardiner, N.Y.) under vacuum at −30 °C. On the day an assay was made, the vacuum flask was allowed to reach room temperature, the vacuum released, and the tissue removed.

Oil Well Techniques. The procedures used for the measurement of enzyme activity in tissues of small sample size have been described by Lowry and Passonneau (1972). If specific cell types were assayed, dissection was done with hand-made scalpels. The dry weight of the samples was determined with a quartz fiber balance. All reactions are done in micro test-tube racks and under a mineral oil interface. The reaction products were amplified to levels appropriate for a fluorometric determination (Model A-4, Farrand Optical Co., New York, N.Y.).

Glycogen Synthetase Assay. Glycogen synthetase activity was determined fluorometrically by a modification of the method of Hornbrook et al. (1966). When a whole individual was assayed, the following procedure was used. Ten microliters of a reaction mixture, containing 50 mM glycylglycine buffer, pH 8.0, 10 mM UDPG, 5 mM EDTA, and 18 mM glycogen (standardized as glucose units), was added to the bottom of an oil well. Glycogen was used in the reaction mixture as a primer for the enzyme. When soluble primer independent activity was measured, the glycogen was omitted. The frozen dried individual was added directly to the reaction mixture in the well. The well was then filled with mineral oil. The Teflon test-tube rack was placed in a plastic petri dish and floated in a 37 °C water bath. Incubation was for 90 min. The reaction was stopped by heating at 90 °C for 10 min.

The reaction mixture was removed from the oil well and was added to a 3-ml test tube containing 50 μl of a UDP reaction mixture. The UDP reaction mixture contained 50 mM glycylglycine buffer, pH 8.0, 0.1 mM phosphoenolpyruvate (PEP), 65 mM KCl, 15 mM $MgCl_2$, 0.04 mM NADH, 15 U/ml of pyruvate kinase (PK), and 5 U/ml of lactate dehydrogenase (LDH). The test tubes were capped and incubated for 40 min at 37 °C. The enzymes and excess NADH were destroyed by adding 50 μl of 0.3 N HCl and by incubating for 10 min at room temperature. One-hundred microliters of 10 N NaOH was added in order to amplify the remaining NAD^+ (Lowry and Passonneau, 1972). The mixture was incubated for 10 min at 60 °C. One milliliter of distilled H_2O was added and fluorescence was measured. UDP standards containing 5.0×10^{-10} and 25×10^{-10} mol were assayed in triplicate each time the assay was done.

The localization of glycogen synthetase activity was investigated in specific cell types by using the microdissection methods as described by Lowry and Passonneau (1972). To the bottom of an oil well was added 0.1 μl of reaction mixture containing 50 mM glycylglycine buffer, pH 8.0, 10 mM UDPG, 5 mM EDTA, 18 mM glycogen, and 0.016% (v) Triton X-100. The Triton X-100 was added to insure release of the entire droplet from the tip of the constriction pipet. When soluble primer independent activity was measured, the glycogen was omitted from the reaction mixture. The tissue section was added to the reaction mixture at the bottom of the well. The reaction mixture was incubated in a 37 °C water bath for 90 min, then heated for 10 min at 90 °C.

The UDP produced was assayed by adding 2.5 μl of a UDP reaction mixture to the reagents in the oil well. The composition of the UDP reaction mixture was as described for the whole organism assay. The well was incubated at 37 °C for 40 min. The reactions were stopped, and excess NADH was destroyed by the addition of 2.5 μl of 0.35 N HCl. The low level of NAD^+ produced was amplified by enzymatic cycling.

The entire reaction volume was removed from the well and added to a 3-ml test tube containing 50 μl of cycling reaction mixture. The cycling reaction mixture contained 100 mM phosphate buffer, pH 7.4, 0.02% bovine serum albumin, 8 mM sodium acetate, 3.5 mM ammonium acetate, 0.2 mM ADP, 2 mM α-ketoglutarate, 2.5 mM mercaptoethanol, 2 mM glyceraldehyde 3-phosphate, 27 U/ml of GAPDH, and 31 U/ml of glutamic dehydrogenase (GDH). The test tubes were capped and incubated for 60 min at 37 °C. Glutamate and 3-phosphoglycerate accumulated as a result of the cycling reactions. After the cycling reactions were completed, 6.5 μl of 3% hydrogen peroxide was added to destroy excess α-ketoglutarate (Matschinsky et al., 1968). The cycling enzymes were denatured by boiling for 3 min.

The glutamate produced in the cycling reaction was assayed by adding 1.0 ml of glutamate reaction mixture, containing 50 mM hydrazine-HCl, pH 9.3, 0.02% albumin, 0.3 mM ADP, 0.4 mM NAD^+, and 5 U/ml of GDH, to the reagents in the test tube. Incubation was for 30 min at room temperature. The fluorescence of the NADH produced was read on a fluorometer equipped with a Corning No. 5840 filter for the incident light and No. 3387 plus 4308 filters for the emitted light.

Attempts to couple the synthetase reaction to the UDP assay were unsuccessful. When 1.5 mg wet weight of cell extract was added to 1.0 ml of 50 mM glycylglycine buffer, pH 8.0, containing 1.2×10^{-5} M NADH, the fluorescence of the NADH rapidly decreased. Because NADH oxidation was probably due to the activity of endogenous NADH oxidases, the use of a coupled assay was invalid.

UDP standards, in triplicate, were carried through all steps of the assay. At least 90% recovery of the standards was obtained. A complete range of standards were included each time the assay was done. Enzyme specific activities are reported as mmol hr^{-1} kg^{-1} dry weight.

End-Product Assays. The end-products of the glycogen synthetase reaction, other than UDP, were also determined. Organisms at the desired stage of development were selected under a stereoscope and were dislodged from the filter paper into a glass homogenizer. The organisms were homogenized, in an ice bath, with a Teflon pestle. The volume of glycogen synthetase reaction mixture was adjusted to give 1 organism/μl. The reaction mixture, containing 50 mM glycylglycine buffer, pH 8.0, 10 mM UDPG, and 5 mM EDTA, was incubated for 90 min at 37 °C. Reactions were stopped by boiling for 3 min, and then were assayed for the possible end-products, UDP, glucose, trehalose, and glycogen.

UDP was assayed according to the method of Hornbrook et al. (1966). A 10-μl aliquot of the incubated homogenate was added to 50 μl of UDP reaction mixture in a 3-ml test tube. The UDP reaction mixture contained 50 mM glycylglycine buffer, pH 8.0, 0.4 mM PEP, 65 mM KCl, 14 mM $MgCl_2$, 0.1 mM NADH, 6.7 U/ml of LDH, and 12.5 U/ml PK. The test tubes were capped and incubated for 40 min at 37 °C. The UDP, produced during the initial incubation of the homogenized

organisms, was converted to NAD^+ as described above. The excess NADH was destroyed by adding 50 μl of 0.35 N HCl and by incubating for 10 min at room temperature. The induced fluorescence of NAD^+ was amplified by adding 100 μl of 10 N NaOH. The mixture was incubated at 60 °C for 10 min. The volume was increased by adding 1.0 ml of distilled H_2O, and the fluorescence was measured. Standards containing 7.8×10^{-10} and 15×10^{-10} mol of UDP were assayed in triplicate.

Glucose was assayed by the method of Lowry and Passonneau (1972). A 100-μl aliquot of incubated homogenate was added to 100 μl of glucose reaction mixture in a 3-ml test tube. The glucose reaction mixture contained 200 mM 4-(*N*-morpholino)ethanesulfonic acid (Mes) buffer, pH 6.5, 2 mM $MgCl_2$, 4.5 mM ATP, 4.5 mM $NADP^+$, 3.5 U/ml of glucose-6-phosphate dehydrogenase (G6PDH), and 5.6 U/ml of hexokinase (HK). Incubations were for 90 min at 37 °C. To stop the reactions and destroy the excess $NADP^+$, 50 μl of 0.6 N NaOH was added. The mixture was incubated for 30 min at 60 °C. A 10-μl aliquot was added to 100 μl of 6 N NaOH containing 0.03% hydrogen peroxide in a 3-ml test tube. This mixture was incubated for 10 min at 60 °C. The volume was increased by adding 1.0 ml of distilled H_2O and the fluorescence measured. Standards containing 7×10^{-10} and 14×10^{-10} mol of glucose were assayed in triplicate.

Trehalose was assayed as described by Jefferson (1975). A 100-μl aliquot of incubated homogenate was added to 100 μl of trehalose reaction mixture in a 3-ml test tube. The trehalose reaction mixture contained 200 mM Mes buffer, pH 6.5, 2 mM $MgCl_2$, 4.5 mM ATP, 4.5 mM $NADP^+$, 3.5 U/ml of G6PDH, 5.6 U/ml of HK, and sufficient trehalase to produce 1.2×10^{-9} mol of glucose/30 min. The reaction mixture was incubated for 90 min at 37 °C. To terminate the reaction and to destroy the excess $NADP^+$, 50 μl of 0.6 N NaOH was added, and incubated 30 min at 60 °C. A 10-μl aliquot was removed to 100 μl of 6 N NaOH containing 0.03% hydrogen peroxide. This mixture was incubated at 60 °C for 10 min. To measure the fluorescence, the volume was increased by adding 1.0 ml of distilled H_2O. Standards, in triplicate, containing 2.5×10^{-10} and 5.0×10^{-10} mol of trehalose were assayed.

Glycogen was assayed according to the method of Passonneau et al. (1967). A 200-μl aliquot of the glycogen synthetase reactions mixture was added to 200 μl of glycogen reaction mixture. The glycogen reaction mixture contained 100 mM imidazole buffer, pH 7.0, 2 mM magnesium acetate, 3.5 mM EDTA, 0.25 mM $NADP^+$, 0.34 mM AMP, 10 mM K_2HPO_4, 0.07% albumin, 1.8 mM dithiothreitol, 1.7×10^{-3} mM glucose 1,6-bisphosphate, 10 U/ml of phosphoglucomutase, 4 U/ml of G6PDH, and 7.7 U/ml of glycogen phosphorylase a. Incubation was for 60 min at 37 °C. The reactions were stopped and excess $NADP^+$ destroyed by adding 100 μl of 6 N NaOH and by incubating for 30 min at 60 °C. A 100-μl aliquot was added to 500 μl of 6 N NaOH containing 0.03% hydrogen peroxide in a 3-ml test tube. The mixture was incubated for 10 min at 60 °C. The final volume was attained by adding 500 μl of distilled H_2O, and the fluorescence was measured. Standards containing 2.5×10^{-9} and 7.0×10^{-9} mol of glycogen were assayed in triplicate.

Results

Glycogen Synthetase Assay. Appropriate controls were included to demonstrate the validity of the glycogen synthetase assay. For example, in the absence of UDPG no activity was observed in cell extracts from any stage of development. When soluble glycogen was omitted from a reaction mixture containing amoeba extract, there was 10% of the activity observed in the presence of added glycogen. This low level of activity may have been due to the presence of endogenous glycogen, present at a sufficient level in the amoeba extract to act as a primer for the enzyme. Alternatively, this activity may be a form of the enzyme which is independent of glycogen as a primer. We will show later in this report that a primer independent activity does occur late in development. The glycogen synthetase assay was linear to at least 10 mg of dry weight/ml of reaction mixture. Likewise, the rate of the reaction was linear for at least 120 min at 37 °C. No differences were found in the activities from fresh homogenates or from lyophilized cell extract. No loss of activity occurred in the lyophilized samples over an 8 month period of storage at −30 °C.

TABLE I: Activity of Glycogen Synthetase during Differentiation of *Dictyostelium discoideum*.

Stage of Development	Time[a] (h)	Sp Act.[b,c] (mmol h^{-1} kg^{-1} dry weight)
Aggregation	8	281[d]
Pseudoplasmodium	12	206
Early culmination	19	136
Culmination	21	125
Late culmination	22	100
Sorocarp	24	99

[a] Time after removing bacterial food source. [b] Determined by measuring UDP produced, as described under Materials and Methods. 10 N NaOH was used for amplification. [c] Glycogen was added as a primer at final concentration of 18 mM (as glucose units). [d] All values are the mean of three replications. Standard deviations were less than 10%.

UDP and glycogen were included as standards for the fluorimetric glycogen synthetase assay. The reaction for both standards was linear over the concentration range used. The concentration of the stock solutions of standards was determined enzymatically in the reaction mixtures described under Materials and Methods.

Both the strong NaOH and cycling methods of amplification were shown to be linear. The NaOH treatment resulted in a tenfold amplification of NAD^+ over the native fluorescence of NADH. Amplification by cycling was achieved with a GAPDH: GDH Unit ratio of 70:30. The number of cycles was dependent upon the levels of the enzymes present.

Glycogen Synthetase Activity during Development. The activity of the enzyme over the time course of differentiation is shown in Table I. A single organism was selected from the lyophilized samples and assayed for glycogen synthetase. Since UDP levels produced during the reaction were near 10^{-10} mol, NaOH amplification was used. Glycogen was added to the reaction mixture as a primer at all stages of development. The assay covered the time period from 8 h, at which time single organisms were first identifiable as aggregates, to 24 h, when the mature sorocarp had developed. During this 16-h period, total glycogen synthetase specific activity decreased from 281 mmol h^{-1} kg^{-1} to 99 mmol h^{-1} kg^{-1}.

Glycogen Synthetase Dependence on Soluble Glycogen Primer during Development. As a control from the glycogen synthetase assay, soluble glycogen was omitted from the reaction mixture at the 18- and 24-h stages. A change in primer dependence of the enzyme was observed between these two stages. A 12-fold increase in enzyme activity was found at preculmination when soluble glycogen was added to the reaction mixture. Sorocarp extract, on the other hand, showed

no appreciable increase in enzyme activity with addition of soluble glycogen to the reaction mixture.

These data indicated a dependence on soluble glycogen primer for enzyme activity during early stages of development that did not exist in the later stages. We then assayed all stages of development both with and without primer added to the reaction mixture. To determine the soluble glycogen dependent activity, the soluble glycogen independent activity was subtracted from the total specific activity. The curve thus produced (Figure 1) showed a decreasing primer dependent activity from 255 mmol h^{-1} kg^{-1} at aggregation to no measurable activity at sorocarp. Conversely, the independent activity was nearly absent in the early stages of development, then increased sharply at 22 h. The units of primer dependent activity lost from culmination to sorocarp were completely recovered as units of primer independent activity. The intersection of the two curves came during the culmination stage of the developmental cycle (i.e., 21–22 h into the developmental process) where the two activities were nearly equal.

End Products of Soluble Glycogen Primer Independent Enzyme Activity. Since in Table I and Figure 1 the assay for soluble glycogen independent glycogen synthetase activity measured the rate of production of UDP from UDPG, the fate of the glucose moiety of UDPG was also investigated. In addition to glycogen, glucose and trehalose were considered as possible co-products with UDP. We assayed for these end products in aliquots taken from a glycogen synthetase reaction mixture containing sorocarp extract. Glycogen, glucose, and trehalose standards were linear with increasing concentrations when assayed in this manner.

Incubation of sorocarp homogenates in the absence of soluble glycogen primer produced no detectable glucose or trehalose. In addition, at least 75% of the UDP produced from UDPG could be accounted for by the synthesis of glycogen. Thus, glycogen was a product of the enzyme activity at the sorocarp stage even in the absence of added soluble glycogen primer. Without soluble glycogen addition to the reaction mixture no net production of glycogen was observed at preculmination over the endogenous level. Nonincubated, boiled blanks of the two stages gave no appreciable difference in glycogen levels from those reactions incubated in the absence of UDPG substrate. Thus, the soluble glycogen primer independent enzyme from sorocarp used UDPG to produce glycogen. However, in the absence of soluble glycogen primer, the enzyme from preculmination extracts showed no activity in the presence of UDPG.

We further investigated whether the enzyme and its product were soluble or particulate. Incubation of sorocarp homogenates in the absence of soluble glycogen, followed by separation of the homogenates into supernatant and 2000*g* pellet (10 min) fractions, resulted in recovery of the majority of bound glycogen product in the pellet fraction. Similar treatment of preculmination extracts resulted in the recovery of soluble glycogen in the supernatant fraction. To determine the soluble or particulate nature of the enzyme, sorocarp homogenates were separated into supernatant and 2000*g* pellet fractions before incubation in the absence of soluble glycogen. The pellet was resuspended in an incubation mixture volume equal to that of the homogenate volume before fractionation. As Table II shows, both the glycogen synthetase activity as well as the bound glycogen produced was found in the pellet fraction. Microscopic examination of the low-spin fractions showed that the pellet consisted primarily of fractured spore cell walls and stalk material. These experiments agree with the previous results of Wright et al. (1968) and suggest that both the enzyme and the product of its activity may become associated with the cell wall in the latter stages of development.

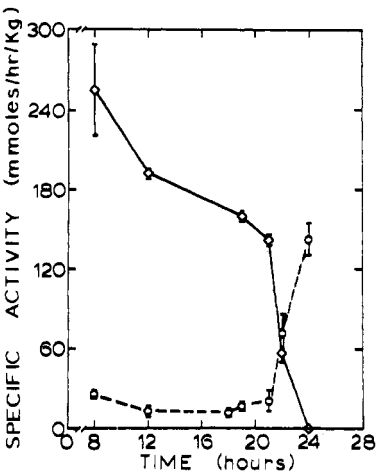

FIGURE 1: Glycogen synthetase activity dependence on soluble glycogen primer during differentiation. (○) Soluble glycogen primer independent activity was assayed with individual organisms in 10 μl of glycogen synthetase reaction mixture minus glycogen, as under Materials and Methods. NaOH was used for amplification. Each point represents the mean and range of three replications. (◇) Soluble glycogen primer dependent activity was calculated by subtracting independent activity from total activity.

TABLE II: Fractionation of Soluble Glycogen Primer Independent Glycogen Synthetase Activity and Glycogen Production in Sorocarp[a,b]

Expt No.	Glycogen Synthetase Act. (10^{-10} mol h^{-1} sorocarp^{-1}) Homogenate	Supernatant	2000*g* Pellet
I	1.23 ± 0.04 (4)[c]	0.0 (4)	1.27 ± 0.12 (4)
II	1.26 ± 0.03 (2)	0.0 (2)	1.69 ± 0.01 (2)
	Level of Glycogen Produced during Incubation[d] (10^{-10} mol/sorocarp)		
I	1.84 ± 0.06 (4)	0.0 (4)	1.89 ± 0.12 (4)
II	1.89 ± 0.05 (2)	0.0 (2)	2.51 ± 0.02 (2)

[a] Glycogen synthetase activity and glycogen production during incubation after fractionation. [b] $NaOH-H_2O_2$ amplification was used. [c] Mean ± standard deviation. Number of replications in parentheses. [d] Incubation was for 90 min at 37 °C.

Glycogen Synthetase Dependence on Soluble Glycogen Primer during Differentiation of Spores. Since bound glycogen and soluble glycogen primer independent glycogen synthetase activity were recovered in the 2000*g* pellet fraction, composed of both stalk and spore cell wall material, glycogen synthetase activity in spore cells free of stalk cells was investigated. With the use of microscalpels, the spore cells were dissected free of the intact stalk. Soluble glycogen primer dependent activity was found in prespore cells of preculmination, but not in the mature spore cells of sorocarp. An eightfold increase in specific activity was found when prespore tissue was incubated in the presence of soluble glycogen. Conversely, there was no difference in the specific activities of sorocarp spore extract incubated with or without soluble glycogen ($P < 0.01$). The preculmination prespore cells showed an increase in specific activity from 19 mmol h^{-1} kg^{-1} to 147 mmol h^{-1} kg^{-1} when soluble glycogen was added to the incubation mixture. The

TABLE III: Dependence of Glycogen Synthetase Specific Activity on Soluble Glycogen Primer during Differentiation of Spore Cells.

		Sp Act.[a] (mmol h^{-1} kg^{-1} dry weight)			
		+Glycogen[c]		−Glycogen	
Stage of Development	Time[b] (h)	I[d]	II[d]	III[d]	IV[d]
Amoeba	0.0	234		49	
		219		92	
Late aggregation	12.0	249		45	
		242		32	
Pseudoplasmodium	14.0	147		27	
				17	
Late pseudoplasmodium	16.0	156		19	
		142		18	
Preculmination	18.5	137		24	
		150		21	
Early culmination	19.5	122	128	15	10
		125	127	–	21
Midculmination	20.5	106	199	21	12
		123	–	11	
Late culmination	22.0	200	142	36	20
		180		43	19
Late culmination	22.5	174	49	194	43
		–	–	153	83
Sorocarp	26.0	207	258	230	260
		216	–	–	–
Late sorocarp	27.5	179	215	88	197
		–	197	136	220

[a] Determined as UDP produced as described under Materials and Methods. Amplification was by enzymatic cycling. [b] Time after removing bacterial food source. [c] Added as a primer at final concentration of 18 mM (as glucose units). [d] Number of stage study.

spore extract from sorocarp showed no change, with the specific activity remaining at about 200 mmol h^{-1} kg^{-1} with or without soluble glycogen in the incubation mixture.

Glycogen Synthetase Activity during Differentiation of Spores. A study of the soluble glycogen primer dependency of glycogen synthetase activity during spore differentiation from initiation of starvation to sorocarp is shown in Table III. The intact stalk was dissected free of spore cells in the latter stages of differentiation. The total specific activity during the time course of spore cell development, as shown in the +glycogen column of Table III, showed a decrease over the 21-h time span from 0 to 21 h. This change was comparable to the two-fold decrease during the time span shown in the +glycogen column in Figure 1 for whole organisms during development. In the absence of soluble glycogen from the incubation mixture, as shown under the −glycogen column in Table III, the activity was low in the early stages of development. A sharp increase in specific activity began during culmination reaching about 200 mmol h^{-1} kg^{-1} at sorocarp. There was a sudden decrease in the soluble primer dependent activity at culmination. At this stage the soluble glycogen primer independent activity was equal to the primer dependent activity. The same results were seen in the assay of synthetase activity in individual organisms using NaOH amplification (Figure 1), with the change over point occurring during culmination. As was the case with individual organisms, the units of soluble glycogen primer dependent activity lost during spore formation were recovered by the units of primer independent activity.

Discussion

In this report we have investigated the nature of the G-6-P independent glycogen synthetase during development of *D. discoideum*. The specific activity of glycogen synthetase was determined, in the absence and presence of soluble glycogen primer, over the time course of development (Table I). The soluble glycogen primer independent specific activity was low from the aggregation stage to preculmination. During culmination and sorocarp construction, the specific activity increased sharply. As evidenced by the increase of primer independent specific activity, with development, the enzyme loses its soluble primer requirement for activity during the culmination and sorocarp stages. Less than 2 h are required for the total conversion in primer requirement.

By subtracting the soluble primer independent glycogen synthetase specific activity from the total specific activity, the specific activity of the soluble primer dependent glycogen synthetase was also determined. As shown in Figure 1, the soluble primer dependent activity decreased throughout the differentiation cycle, with the sharpest drop coinciding with the change in primer requirement at culmination.

The apparent activity of a soluble primer independent glycogen synthetase during the terminal stages of development prompted an investigation into the nature of the enzyme product. We found that glycogen was the major product of the primer independent activity at the sorocarp stage. Furthermore, the enzyme and its product can be recovered by sedimenting with low-speed centrifugation. These results are consistent with those reported by Wright et al. (1968), thus providing independent evidence that during development, the enzyme loses its requirement for soluble primer by becoming associated with cell-wall material. Although this result occurs only at a specific stage of development, the possibility exists that the change in the solubility of the synthetase during differentiation might be artifactual, i.e., a result of adsorption to particulate material after cell breakage.

Regulation of Glycogen Synthetase during Differentiation. There are several factors related to metabolite level that can account for a kinetic situation favorable for insoluble glycogen synthesis during spore formation. Gezelius and Wright (1965) showed that the level of inorganic phosphate (P_i) increased 20-fold during the developmental stages from myxamoebae to sorocarp. The increase in P_i concentration was from 3 to 50 mM. It was substrate specific for UDPG. The apparent K_m for UDPG was 1.3×10^{-3} M in the presence or absence of G-6-P. The glycogen product of the enzyme activity was found associated with the cell-wall material. Wright et al. (1966) showed that soluble glycogen synthetase obtained from myxamoebae will adhere to cell-wall material prepared from a later stage of development. The enzyme was eluted from the cell-wall material with glycogen. Subsequently, the cell-wall material was used as a source of both enzyme and primer to synthesize insoluble cell-wall associated glycogen from UDPG.

Using crude extracts prepared from various stages of development, Rosness et al. (1971) found a G-6-P-dependent (D) form and a G-6-P-independent (I) form of the enzyme. I activity decreased from myxamoebae stage to the culmination stage of development, whereas D activity increased during the same period of development.

Regulation of Glycogen Synthetase during Differentiation. There are several factors related to metabolite level that can account for a kinetic situation favorable for insoluble glycogen synthesis during spore formation. Gezelius and Wright (1965) showed that the level of P_i increased 20-fold during the de-

velopmental stages from myxamoebae to sorocarp. The increase in P_i concentration from 3 to 50 mM would be sufficient to inhibit soluble glycogen synthetase by 80% and cell-wall glycogen synthetase by 50% (Wright, 1973). The known increase of G-6-P and UDPG from myxamoebae stage to culmination has multiple effects on glycogen synthetase (Wright, 1966; Hames and Ashworth, 1974b). The increased concentration of G-6-P lowers the soluble (D) glycogen synthetase K_m for UDPG. Conversely, the increased level of UDPG decreases the requirement for G-6-P (Wright and Dahlberg, 1967). Thus, the rising concentration of G-6-P prior to culmination would favor the activity of soluble (D) glycogen synthetase. In the presence of G-6-P and limiting UDPG concentrations found in the early stages of development, soluble (D) glycogen synthetase could favorably compete for the available UDPG. Later in development, when G-6-P levels decrease to low concentrations, the competition for UDPG might favor production of cell-wall glycogen, since the insoluble glycogen synthetase is independent of G-6-P for activity (Wright et al., 1968). Further, trehalose, formed from G-6-P and UDPG, stimulates cell-wall glycogen by some unknown mechanism (Wright, 1973).

It is likely that the association of a major portion of the soluble glycogen synthetase with the spore cell wall protects it from the inhibitory effects of P_i, as well as alters its dependence on G-6-P. As the P_i concentration increases during differentiation, glycogen phosphorylase activity may be increased, while the activity of glycogen synthetase may be decreased. These changes in enzyme activities would result in the degradation of glycogen. In *D. discoideum,* as with other systems, soluble glycogen synthetase may be combined with soluble glycogen particles (Meyer et al., 1970; McVerry and Kim, 1974). With the degradation of the glycogen particle, glycogen synthetase would become soluble. The soluble enzyme may then be translocated to the developing spore cell wall. The cell wall or glycogen bound to the cell wall would provide a primer source for the production of insoluble glycogen.

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