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Protein Synthesis During the Differentiation of Sporangia in the Water Mold *Achlya*

WILLIAM E. TIMBERLAKE, LARRY McDOWELL, JOHN CHENEY, AND DAVID H. GRIFFIN

Department of Botany, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210

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During the synchronous differentiation of sporangia in the absence of added nutrients, the water mold *Achlya bisexualis* (Coker and Couch) actively synthesized protein. Inhibition of protein synthesis at any time during the sporulation process completely inhibited further differentiation. Large changes in the rate of radioactive amino acid uptake resulted in changes in the specific activity of the cellular amino acid pool. The rate of protein synthesis was calculated from the amino acid pool specific activity and the incorporation of isotope into protein. During the 1st h after induction of the sporulation process, the rate of protein synthesis increased to two times the initial value. The amino acid precursors for this synthesis were supplied by the degradation of preexisting protein. Proteolytic enzyme activity assayed in vitro increased in proportion to the in vivo rates of protein synthesis and degradation. Differentiation was accompanied by a slight decline in dry weight of the mycelium as well as by a decrease in the protein content, whereas the relative size of the amino acid pools remained constant.

The control of the induction of the differentiation of sporangia and subsequent formation and release of spores has been well delineated for the aquatic oömycete *Achlya bisexualis* (3). Furthermore, the morphological changes associated with differentiation have been examined and correlated with certain biochemical events (4). The high degree of synchrony associated with the differentiation of whole cultures of *Achlya*, the ability to grow vegetative cultures under defined conditions, and the ease with which both growing and differentiating cultures are handled experimentally make *Achlya* an ideal organism for the study of physiological and biochemical events associated with the production of asexual reproductive structures.

The fact that induction of the sporulation process in *Achlya* involves the removal of the supply of organic nutrients (3) shows that the continued synthesis of macromolecules must depend on the utilization of cellular stores formed during vegetative growth. Although the incorporation of radioactively labeled amino acids into protein during the differentiation of reproductive structures by fungi under starvation conditions has been shown (7, 9), little is known about the actual rate of synthesis or the origin of the cellular precursor pools under these conditions. The objective of this paper is to show that *Achlya* actively synthesizes protein during differentiation at a rate considerably greater than that observed at the time of initiation of the sporulation sequence and that the source for the amino acids required for this synthesis is the degradation and reutilization of preexisting protein.

MATERIALS AND METHODS

Culturing and induction of sporulation. *Achlya bisexualis* (65-1) was grown as described by Griffin and Breuker (4) in a medium containing 5.00 g of D-glucose, 1.25 g of peptone, and 1.25 g of yeast extract per liter of glass-distilled water (PYG). Medium (80 ml) in 250-ml Erlenmeyer flasks was inoculated with 2×10^6 spores and incubated at 30 C on a rotary shaker at 150 rpm for 16 h. Under these conditions, the fungus grows as a uniform suspension without forming pellets. To induce synchronous sporulation, these were harvested and washed with 200 ml of 0.5 mM $CaCl_2$ on a sintered glass filter with suction, taking care not to draw air through the mycelium. These mycelia were then transferred into 1 liter of 0.5 mM $CaCl_2$ in a 2-liter Erlenmeyer flask and incubated at 30 C on a rotary shaker at 60 rpm. Under these conditions, the differentiation of sporangia and spores was 20 to 30% faster than previously reported (4). Cultures were examined microscopically just prior to induction and at 1-h intervals during differentiation for bacterial contamination and normal morphological development of reproductive structures.

Analytical techniques. Dry weight, protein concentration, and amino acid pool size were measured as a function of time after the induction of sporulation. For the measurement of dry weight, 100-ml samples of the $CaCl_2$ suspension were harvested on preweighed Whatman no. 1 filter papers, washed with 0.5 mM $CaCl_2$, and dried at 60 C. Amino acid and protein measurements were performed by harvesting 50-ml samples as above and placing the washed mycelium in 2 ml of ice-cold 1.5 N formic acid. Preliminary experiments showed that this technique was as effective as cold 10% (wt/vol) trichloroacetic acid or 1 N perchloric acid in precipitating proteins within the mycelium. After 1 h each sample was filtered through a glass fiber filter (Reeve-Angel 934 AH) and washed with 1 ml of cold 1.5 N formic acid. The filtrate was collected and evaporated to dryness at 60 C. The residue retained by the filter was placed in 5 ml of 10% (wt/vol) trichloroacetic acid and extracted in a boiling water bath for 15 min. The hot acid-insoluble material was collected on a glass fiber filter and washed with 30 ml of cold trichloroacetic acid and 20 ml of ice-cold ethanol. The residue was then extracted with 2 ml of 1 N NH_4OH at 80 C. Insoluble material was removed by filtration through a glass fiber filter, and the filtrate was dried at 60 C.

Both the cold acid-soluble and hot acid-insoluble extracts were dissolved in 0.2 ml of glass-distilled water and assayed for Folin phenol reactive material by a modification of the procedure of Lowry et al. (4, 6). The cold acid-soluble extract was also assayed for ninhydrin reactive material by using the method of Rosen (8). Standard samples of bovine serum albumin and casein hydrolysate were measured by the same procedures and used to estimate the amount of acid-insoluble protein and acid-soluble amino acids. At all stages of differentiation, the estimates of amino acid pool size by the methods of Lowry and of Rosen agreed within 10%.

Total nucleic acid and protein content of the mycelium before induction and in the spores produced by an equal amount of mycelium was determined by a modification of the procedure of Smillie and Krotkov (10) as described by Griffin and Breuker (4). For these determinations, 10 mg of exponentially growing hyphae were collected by filtration, washed, and killed in cold 10% trichloroacetic acid. Another 10-mg sample was collected and induced as above. After essentially all the sporangia had discharged, the mycelium was removed by filtration on Mirra cloth and washed extensively with 0.5 mM $CaCl_2$. The spores were collected from the filtrate by centrifugation for 10 min at 5,000 $\times$ g and were placed in trichloroacetic acid as before. The samples were then processed in parallel. Protein was quantitated by the method of Lowry et al. (6) and nucleic acid by absorbancy at 260 nm.

Measurement of the rates of amino acid uptake, incorporation, and protein synthesis. To measure the rate of uptake of amino acids, 0.1 μCi of ^{3}H-amino acid mixture per ml (Schwarz-Mann, yeast profile) was added to the $CaCl_2$ suspension at intervals during the sporulation sequence. Samples (40 ml) were collected on glass fiber filters, washed with 50 ml of 0.5 mM $CaCl_2$, and dried at 60 C. These were weighed, and radioactivity was determined in 5 ml of a toluene-based scintillation fluid containing 3.2 g of 2,5-diphenyloxazole (PPO) and 0.3 g of dimethyl 1,4-bis-2-(5-phenyloxazoly benzene (POPOP) per liter and 0.05 ml of NCS solubilizer (Amersham-Searle) on a Packard Tri-Carb 3375 scintillation counter.

For determination of incorporation and protein synthetic rate, cultures were pulse labeled with ^{3}H-amino acids as above, and amino acids and protein were prepared as previously described. Amino acid content was determined as described under the paragraph heading "analytical techniques," samples of each preparation were dried in scintillation vials, and radioactivity was determined in the toluene-PPO-POPOP-NCS scintillation fluid. The data were used to calculate the specific activity of the amino acid pools.

Measurement of protein degradation, turnover, and proteolytic enzymes. Protein was labeled in growing cultures by the addition of 2 μCi of ^{14}C-amino acid mixture per 80 ml of medium (Schwarz-Mann, yeast profile) at 13 h after inoculation, and incubation at 30 C continued for 2 h. The culture was then harvested aseptically on sintered glass with suction, washed with 100 ml of unlabeled PYG, and resuspended in 80 ml of fresh unlabeled medium. After one more hour of incubation, the culture was harvested and induced. The net loss of protein, the change in the specific radioactivity of prelabeled protein, and the specific rate of protein synthesis were used to calculate the in vivo rate of protein degradation and fraction of amino acids degraded from preexisting protein which were utilized for new synthesis.

An estimate of the proteolytic enzyme content of the differentiating cultures was obtained by homogenizing a 200-ml sample harvested as above in 10 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM $CaCl_2$, pH 7.5 at 4 C, for 1 min at top speed in an Omnimixer (Sorvall, Inc., Norwalk, Conn.) with glass beads. Cell walls were removed by centrifugation, and protein concentration was determined by absorbancy at 260 and 280 nm and adjusted to 0.7 mg/ml. In vitro protease activity was determined by the hydrolysis and concomitant increase in absorbancy at 247 nm of *p*-tosyl-L-arginine methyl ester, by the hydrolysis of N-benzoyl-L-tyrosine ethyl ester and increase in absorbancy at 256 nm, as described by Hummel (5), as well as by the degradation of bovine serum albumin. In this procedure, 5 mg of bovine serum albumin was incubated with 100 μg of protein from the cell extract in 1 ml of 10 mM Tris, 1 mM $CaCl_2$, pH 7.9 at 25 C for 24 h at 25 C. The reaction was stopped by adding 1 ml of cold 10% trichloroacetic acid. The protein precipitate was removed by centrifugation, and the absorbancy of the supernatant fluid at 280 nm was measured against a zero time control.

Effect of cycloheximide on sporulation. Cycloheximide was added to induced cultures at 1-h intervals starting at 0 h postinduction to a final concentration of 2.5 μM, the minimal concentration which completely inhibits vegetative growth and protein synthesis (W. E. Timberlake, unpublished

data). At 5.25 h, the cultures were examined microscopically for normal development.

Reagents. *Para*-tosyl-L-arginine methyl ester hydrochloride and N-benzoyl-L-tyrosine ethyl ester were obtained from Schwarz-Mann; Trizma base and PPO were from Sigma; Folin phenol reagent was from Fisher; dimethyl POPOP was from Amersham-Searle; and cycloheximide was from Calbiochem. The composition and specific radioactivity of the ^{3}H and ^{14}C L-amino acid mixtures are listed in Table 1. All other chemicals were reagent grade or better.

RESULTS

Inhibition of the differentiation of sporangia and spores by cycloheximide. The addition of 2.5 μM cycloheximide, an inhibitor of protein synthesis in eukaryotes (2, 13), to sporulating cultures arrested the developmental process at the stage during which the antibiotic was administered (Table 2). Complete inhibition of development was observed even in the 5-h treatment when sporangia were actively discharging spores. The addition of the drug stopped the release of spores by undischarged, but fully cleaved, papillate sporangia. Control cultures incubated in the absence of cycloheximide differentiated sporangia and released spores as described previously (4).

Changes in dry weight, protein content, and amino acid pools during differentiation. Because the induction of sporulation includes the removal of all exogenous organic nutrients, the developmental process must be maintained by the utilization of endogenous materials formed during growth. That cycloheximide inhibited differentiation at all stages indicates that protein synthesis, a process utilizing energy and amino acids, was required. We were therefore interested in whether there were changes in the dry weight, protein content, or amino acid pools during the differentiation process.

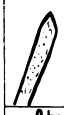
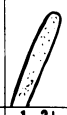
There was a small decline in the dry weight of the fungus as well as a decrease in protein content of the mycelium (Fig. 1). On the other hand, the acid-extractable amino acid content of the differentiating mycelium remained constant throughout the developmental sequence. There were no detectable amino acids in the 0.5 mM $CaCl_2$ wash when it was assayed by the techniques described in the Materials and Methods section, which showed that there is little or no loss of intracellular amino acids due to the procedure used.

To estimate the contribution of vegetative protein and nucleic acid to spore formation, the total cellular content of these components was measured in the mycelium before induction and in the spores as described in the Materials and Methods section. The results of this experiment are shown in Table 3 and demonstrate that the amount of these components found in the spores

TABLE 1. *Composition and specific radioactivity of ^{3}H and ^{14}C L-amino acid mixtures*

Amino acid	μCi/mCi of mixture	^{3}H specific activity (Ci/mmole)	^{14}C specific activity (mCi/mmole)
Alanine	70	3.5	156
Arginine	60	2.78	312
Aspartic acid	100	1.0	210
Glutamic acid	120	15	265
Glycine	50	20	104
Histidine	20	3.5	312
Isoleucine	60	39	312
Leucine	100	58	312
Lysine	80	30	312
Phenylalanine	70	7.0	455
Proline	45	27	258
Serine	50	1.2	158
Threonine	55	0.82	208
Tyrosine	45	52	460
Valine	75	6.0	260

TABLE 2. *Inhibition of sporulation by cycloheximide*[a]

CYCLOHEXIMIDE ADDED AT HOURS AFTER INDUCTION	STAGES OBSERVED AT 5.25 HR AND TIME NORMALLY FORMED					
	growing	rounding	refractile	septate	papillate	discharged
						
	0 hr	1–2 hr	3 hr	4 hr	4.5 hr	5 hr
NONE					X	X
0	X					
1	X	X				
2	X	X				
3		X	X			
4			X	X		
5					X	X

[a] Cycloheximide was added to differentiating cultures at the times shown to a final concentration which completely inhibited protein synthesis. At 5.25 h after induction, the cultures were scored for rounding of hyphal tips, refractility, septum and papilla formation, and release of spores. X denotes the stages observed at 5.25 h for each treatment. The time at which each of these stages normally occurs is shown.

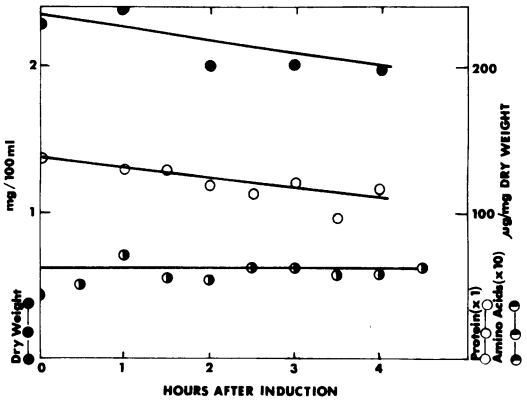


FIG. 1. *Dry weight, protein and amino acid content of the mycelium during differentiation. Samples for each determination were collected and processed as described under "analytical techniques" in the Materials and Methods section. The observed amino acid concentrations have been multiplied by 10 and presented on the same scale as protein content.*

is a large fraction of that in the vegetative mycelium from which the spores were formed. These data agree with our observations that under the conditions described virtually all of the hyphal tips are converted to sporangia, and a large fraction of the cytoplasm in the hyphae migrates into the differentiating tips.

Rate of uptake and incorporation of radioactive amino acids. Because our data indicated that protein synthesis was required for the normal development of sporangia and spores, that protein was degraded during the process, and that amino acid pools were not depleted, we decided to measure the in vivo rates of protein synthesis, degradation, and turnover. It has been shown that the rate of uptake of some compounds by water molds changes during sporulation and that these changes may influence the observed rates of radioactive precursor incorporation into macromolecules (7). Any such changes in amino acid transport would of course complicate the measurement of in vivo rates of protein synthesis by the incorporation of radioactively labeled amino acids into protein.

Figure 2 shows the results of experiments measuring the rates of uptake and incorporation of ^{3}H-amino acids during differentiation, as measured over a 30-min time course at each point indicated. After induction there was a rapid and large increase in the rate of uptake and incorporation into protein of the isotope. The maximum rate of uptake occurred at about 2 h postinduction, whereas the maximum rate of incorporation was at about 3.5 h. After reaching the maximum values, both uptake and incorporation rates declined, but never returned to the rates observed at zero time. The extreme change in the rate of amino acid uptake indicates that the changes in incorporation into protein may not accurately reflect changes in the synthetic rate, but rather differences in the specific radioactivity of the amino acid pools.

Rates of protein synthesis. To determine the actual in vivo rate of protein synthesis during differentiation, the specific radioactivity of the amino acid pools was estimated in parallel with the incorporation of ^{3}H-amino acids into protein, as described in the Materials and Methods section, and was used to calculate synthetic rates as micrograms of protein synthesized per minute per mg (dry weight) (Fig. 3). At zero time the rate of protein synthesis was

TABLE 3. *Nucleic acids and protein in vegetative mycelium and spores*

Substance	Vegetative mycelium[a]	Spores[a]	% In spores[b]
Nucleic acid	106	68	64
Protein	180	83	46

[a] The data are shown as μg/mg (dry weight) of mycelium. For the spores, the values are calculated on the basis of the dry weight of mycelium used for induction.

[b] Percent in spores is the amount of either fraction remaining in the spores over the amount in the vegetative mycelium X 100.

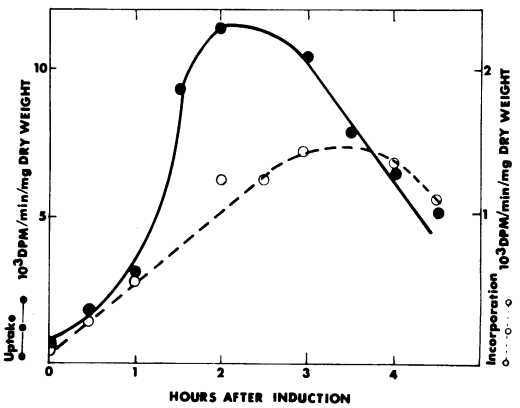


FIG. 2. *The rates of uptake and incorporation into protein of ^{3}H-amino acids as a function of time after induction of the sporulation process. At intervals during the differentiation of sporangia, ^{3}H-amino acids were added to cultures, and samples were collected and treated as described in the Materials and Methods section. The initial rates of incorporation and uptake were calculated from the time course with points at 0, 10, 20, and 30 min after the addition of isotope and are shown as per minute values.*

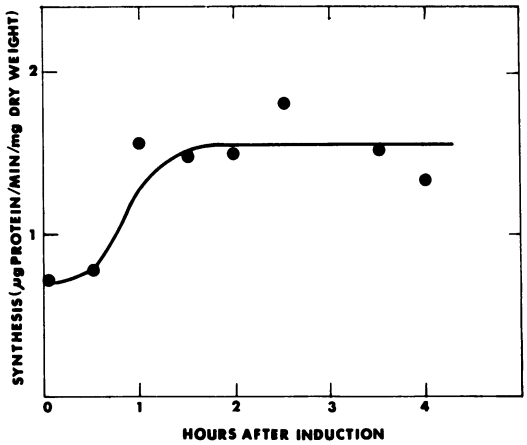


FIG. 3. *Rate of in vivo protein synthesis during differentiation. The specific radioactivity of the amino acid pools and the incorporation of label into protein were determined by collecting and processing samples as described in the Materials and Methods section at 0, 10, 20, and 30 min after the addition of isotope. The time course of specific radioactivity of the amino acid pools and incorporation into protein were used to calculate the specific rate of protein synthesis.*

0.72 μg per min per mg (dry weight). By using the protein content of the mycelium, the protein doubling time was determined to be 3.1 h, a value in close agreement with the dry weight doubling time of noninduced cultures on PYG medium (2.8 h). Because it has been shown that protein concentration as a function of dry weight is constant in exponentially growing cultures of *Achlya* (D. H. Griffin, unpublished data), these calculations indicate that the methods employed to determine in vivo rates of protein synthesis are valid.

During the 1st h after induction of the sporulation sequence, the rate of protein synthesis rapidly increased to a value two times greater than that observed at zero time. After this initial increase, the rate of synthesis remained constant throughout the rest of the developmental sequence (Fig. 3).

Rates of protein degradation and turnover. The fact that protein synthesis proceeds at a rapid rate during sporulation, whereas there is a decline in total protein content of the mycelium and the precursor pools are not depleted, shows that protein degradation must also occur at a rapid rate. Amino acids so released could be returned to the precursor pools and incorporated into new protein. To determine the rate of turnover, protein was labeled with ^{14}C-amino acids, as described in the Materials and Methods section, during the growth phase of the organism, and protein radioactivity was measured in the absence of added label during differentiation (Fig. 4). During the sporulation process there was a gradual decline in the specific radioactivity of the protein, which resulted in a 24% decrease in specific activity at the time of spore discharge.

By using the protein content of the mycelium, the rate of protein synthesis, and the ^{14}C prelabeled protein specific activity, the in vivo rate of protein degradation and the fraction of the amino acids released, which were utilized in de novo protein synthesis, were calculated. The 10-min rate of protein degradation, the fraction of degraded protein reutilized in synthesis, and the fraction of the cellular protein turned over are shown as a function of time after induction (Table 4). Both the rate of protein degradation and turnover increased rapidly during the 1st h after induction, and were then maintained at a high, constant value.

In another experiment, cultures were labeled during growth with ^{14}C-amino acids, induced, and pulse labeled with ^{3}H-amino acids for 10 min at 0.5-h intervals during sporulation. After pulse labeling, the cultures were harvested by filtration and suspended in fresh $CaCl_2$ with 50 μg of casein hydrolysate per ml, and 2.5 μM cycloheximide was added to halt further incorporation of isotope into protein. Protein radioactivity was measured immediately after the "chase-inhibition" was started and at 15, 30, and 45 min. The ratio of ^{14}C to ^{3}H radioactivity

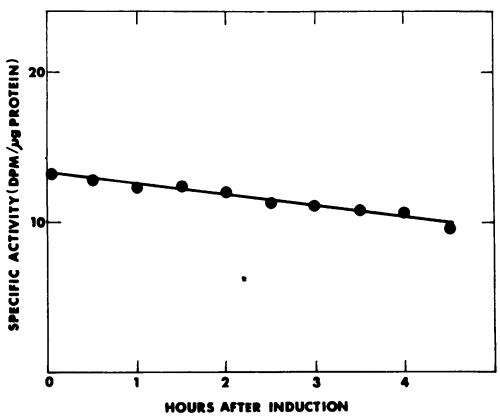


FIG. 4. *The loss of radioactivity from protein labeled with ^{14}C-amino acids during the growth of the organism. Protein was prelabeled during growth as described in the Materials and Methods section. At intervals during the differentiation of sporangia, 50-ml samples were taken, and the amount of protein and protein radioactivity was determined. These values were used to calculate the protein-specific radioactivity shown in the figure.*

TABLE 4. *Summary of protein degradation and turnover during the differentiation of sporangia*

Hours after induction	Protein degraded per 10 min per mg dry weight (μg)[a]	Reutilization (%)[b]	Protein turned over per 10 min (%)[c]
0	8.4	85.9	5.3
1	14.4	91.6	10.2
2	16.2	92.5	12.3
3	16.2	92.4	12.8
4	16.2	92.7	13.9

[a] The rate of protein degradation is equal to the rate of synthesis plus the rate of loss of protein as shown in Fig. 1.

[b] Reutilization was calculated from the actual loss of ^{14}C radioactivity from prelabeled protein and the predicted loss of radioactivity as determined from Fig. 1 and 4.

[c] The turnover value is the product of the degradation and the reutilization divided by the protein content of the mycelium.

was calculated to ascertain whether there was preferential degradation of either prelabeled or newly labeled protein. At all times during differentiation the isotope ratios remained constant after the "chase-inhibition" was begun, showing that degradation was general.

Measurement of protease activity in vitro. Protease activity was assayed in three different ways from whole-cell extracts prepared from differentiating cultures at 1-h intervals after induction (Table 5). Protease specific activity measured under each condition paralleled in vivo rates of protein degradation and synthesis, providing further support for the in vivo measurements.

DISCUSSION

The water mold *Achlya* provides a valuable system for the study of the biochemical events involved in the development of asexual reproductive structures. The differentiation of sporangia of whole cultures can be induced by simple techniques, proceeds with a high degree of synchrony, and has distinct changes in morphology which can be correlated with biochemical changes. Furthermore, our data indicate that a large fraction of the mycelium contributes to the formation of spores. This means that it is unlikely that biochemical events associated with the developmental process will be masked by uninvolved processes. The developmental sequence has been well documented under a variety of conditions (3, 11).

As was shown by Griffin and Breuker (4), ribonucleic acid (RNA) synthesis is required for the normal sequence of developmental events in this organism. We have shown that the administration of cycloheximide at any time during differentiation completely inhibits further development. Although it is generally assumed that cycloheximide is a specific inhibitor of eukaryotic protein synthesis, Cameron and LeJohn (1) have shown that this antibiotic effectively inhibits both protein and RNA synthesis in *Achlya*. Thus, the cycloheximide data do not conclusively show that protein synthesis is required for the completion of the developmental sequence resulting in mature sporangia and spores. However, as shown here, protein synthesis proceeds at a rapid rate throughout the process, indicating that it is an important function during normal development.

Although the whole process of differentiation occurs in the absence of exogenous nutrients, *Achlya* has the ability to convert endogenous stores to forms needed for the formation of reproductive structures. The high rate of protein turnover shown by this study is an excellent example of the efficiency of reutilization by this organism. Protein turnover during the differentiation of spores has been demonstrated in bacteria (12) and the slime mold *Physarum polycephalum* (9), a primitive eukaryote. The protein turnover observed in our experiments was similar to that observed in bacterial spore formation in that there was general degradation of proteins rather than the preferential degradation of proteins formed during the growth phase as observed in *Physarum*. However, the rate of turnover during differentiation is extremely

TABLE 5. *Protease specific activity during differentiation*[a]

Hours after induction	TAME[b]	BTEE[c]	BSA[d]
0	0.204	0.75	0.307
1	0.461	1.77	0.621
2	0.374	1.45	0.672
3	0.503	1.93	0.704
4	0.433	1.73	0.602

[a] Protease enzymes were extracted and assayed in vitro during differentiation as described in the Materials and Methods section. All data are presented as units per milligram of protein.

[b] One unit is 1 μmol of *p*-tosyl-L-arginine methyl ester hydrolyzed per minute at 25 C.

[c] One unit is 1 μmol of N-benzoyl-L-tyrosine ethyl ester hydrolyzed per min at 25 C.

[d] One unit is equal to a 280 nm absorbancy of trichloroacetic acid-soluble hydrolysis products of 0.001 per min per ml at 25 C.

high, i.e., almost four turnovers during the development of sporangia, or about 75% per hour. Spudich and Kornberg (12) reported a turnover rate of 18% per hour during the formation of spores of *Bacillus subtilis*, with a developmental time of about 14 h. Thus, although the rate of turnover in *Achlya* is 4.2 times greater than that of *B. subtilis*, its developmental time is 3.1 times shorter. Because the value presented by Spudich and Kornberg is minimal, there is reasonable agreement between the total number of turnovers during the developmental times. The decrease in dry weight during differentiation could be the result of the oxidation of cellular storage products used for the production of high-energy compounds needed for both protein and RNA synthesis.

The water mold *A. bisexualis* synthesized protein at a high rate during the differentiation of sporangia under starvation conditions. The actual rate of synthesis doubled soon after the induction of the sporulation process. The precursors for this synthesis were supplied by rapid degradation and turnover of preexisting protein. Furthermore, changes in the capacity of the cells to transport radioactive amino acids during differentiation made it impossible to determine in vivo rates of synthesis or degradation of protein without determining the specific activity of the precursor pools. Further studies on the biochemical events associated with sporulation should provide valuable information regarding the control of development in eukaryotes.

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