Rates of Accumulation of Glycosidase Activities during Growth and Differentiation of *Dictyostelium discoideum*

By DALE EVERY* and JOHN M. ASHWORTH†

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

(Received 19 August 1974)

1. The rates of accumulation (enzyme units/h per 10^8 cells) of a number of glycosidase activities were studied in *Dictyostelium discoideum* cells during the growth and differentiation phases of this organism's life cycle. 2. The rates of accumulation of the enzymes β -N-acetylglucosaminidase, α -glucosidase and β -galactosidase remain unchanged during the growth and early differentiation phases. 3. The considerable changes in specific activity of the enzymes which occur in the early differentiation phase are due to the massive loss of total cellular protein which occurs at this time. 4. Significant alterations can occur in the rates of accumulation of α -mannosidase during both the growth and differentiation phases, and since, on the onset of differentiation, β -glucosidase activity is excreted and degraded, the rate of accumulation of this enzyme differs in the growth and differentiation phases. 5. The characteristic rates of accumulation of all these glycosidases change markedly with changes in the growth conditions of the myxamoebae, and thus these rates of synthesis must be regulated independently; however, addition of cyclic AMP to the growth medium has no effect on them.

The life cycle of the cellular slime mould *Dictyostelium discoideum* consists of two distinct phases which are mutually exclusive in the sense that cell growth and cell division are confined to the feeding phase, when the organism exists in the form of solitary amoeboid cells (myxamoebae), whereas morphogenesis and cell differentiation occur in the absence of growth and nutrients during the subsequent, multicellular, developmental phase [for a detailed description of the life cycle see Bonner (1967)]. There is evidence for the belief that different genes are active at different phases of this life cycle (Loomis, 1969b), and considerable attention has been focused on changes in the specific activity of a number of enzymes in the belief that these changes might reflect differential changes in gene activity. In particular changes in the activity of the glycosidases β -N-acetylglucosaminidase, β -glucosidase and α -mannosidase have been studied during development of the cellular slime mould *D. discoideum* strain NC-4 (Loomis, 1969a,b, 1970; Coston & Loomis, 1969) and during growth and development of *D. discoideum* strain Ax-2 (Wiener & Ashworth, 1970; Quance & Ashworth, 1972; Ashworth & Quance, 1972). However, it is possible that these changes in specific activity reflect changes in the rate of total protein turnover, enzyme degradation or

enzyme activation, rather than changes in the rate of enzyme synthesis itself (Gustafson & Wright, 1972).

In the present paper we examine the changes in activity of various glycosidases during growth and differentiation of the cellular slime mould under a variety of conditions. From these results quantitative determinations have been made of the actual rate of accumulation of assayable enzyme activity, k_a (enzyme units/unit time per cell), which gives a clearer indication of possible changes in the rate of enzyme synthesis than do specific-activity measurements. The enzymes studied are β -N-acetylglucosaminidase (EC 3.2.1.30), α -mannosidase (EC 3.2.1.24), β -glucosidase (EC 3.2.1.21), α -glucosidase (EC 3.2.1.20) and β -galactosidase (EC 3.2.1.23). In the following paper the k_a values are used to calculate the absolute rate of synthesis *de novo* of β -N-acetylglucosaminidase and α -glucosidase (Every & Ashworth, 1975).

Materials and Methods

Materials

p-Nitrophenyl glycosides were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and cyclic AMP was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. All other chemicals were of the highest purity available and were the products of either BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

* Present address: Wallaceville Animal Research Centre, Private Bag, Upper Hutt, New Zealand.

† To whom reprint requests should be sent. Present address: Department of Biology, University of Essex, Colchester CO4 3SQ, U.K.

Methods

Organism and culture conditions. *D. discoideum* strain Ax-2 (A.T.C.C. 24397) was grown either in axenic medium containing 86mM-glucose as described by Watts & Ashworth (1970) or in 0.0167M-sodium phosphate buffer, pH 6.0, suspension with *Escherichia coli* B/r as a nutrient source as described by Gerisch (1960). The myxamoebae were grown either in 60–80 ml portions of medium in 250 ml Erlenmeyer flasks or in 700 ml portions of medium in 2-litre Erlenmeyer flasks. Cell densities were determined by counting with a haemocytometer. However, before counting low cell densities of myxamoebae growing in suspension with *E. coli* it was necessary to remove bacteria by centrifugation at 400g for 5 min and to wash the myxamoebae three times with ice-cold 0.0167M-sodium phosphate buffer, pH 6.0. Washed cells and particle-free growth medium were stored at -20°C for subsequent protein determination by the method of Lowry *et al.* (1951) with bovine serum albumin as standard and/or for enzyme assays. *D. discoideum* strain NC-4 was grown on agar plates in association with *Klebsiella aerogenes* as nutrient (Sussman, 1966).

Development of *D. discoideum*. Washed myxamoebae from the exponential phase of growth (2×10^6 – 4×10^6 cells/ml of culture) were suspended in ice-cold water and deposited on Millipore filters (47 mm diam., catalogue no. AABP 04700) supported on Millipore absorbent pads (catalogue no. AP 1004700), containing KCl, MgCl_2 and 50mM-sodium-potassium phosphate buffer, pH 6.5, as described by Sussman (1966). Cell densities of 2.5×10^7 cells per filter for axenically grown cells and 5×10^7 cells per filter for bacterially grown cells were used. These filters were incubated in a humid dark environment at 22 – 23°C . Cells were harvested with 4 ml of water and the extracellular medium was collected by squeezing the Millipore absorbent pads. Samples were stored frozen before assaying for protein and enzyme.

Myxamoebae were also allowed to develop in suspension to the aggregation competent stage as described by Gerisch (1960) and Malchow *et al.* (1972). Washed myxamoebae from the exponential growth phase (2.4×10^6 cells/ml of culture) were suspended in 0.0167M-sodium phosphate buffer, pH 6.0, at 10^7 cells/ml and shaken at 22 – 23°C in Erlenmeyer flasks on a rotary shaker with an eccentric rotation of 3 cm at 150–170 rev./min. To check qualitatively for the development of aggregation competence the 'cover-slip test' developed by Gerisch (1962) was used. Cells and suspending medium were separated by centrifugation at 1000g for 5 min and stored frozen before assay for protein and enzyme.

Enzyme assays. Samples of growing cells and of

cells shaken in phosphate buffer were disrupted by the addition of Triton X-100 to a final concentration of 0.1%. Cells allowed to differentiate on a solid substratum were disrupted by sonication. The glycosidases were assayed as described by Every & Ashworth (1973) in 0.02M-sodium phosphate–0.02M-sodium citrate buffer at pH 5 for β -N-acetylglucosaminidase, at pH 4 for α -mannosidase and at pH 3.5 for β -glucosidase, α -glucosidase and β -galactosidase. The enzyme preparations were incubated at 35°C for 5–60 min in buffer containing the appropriate *p*-nitrophenyl glycoside (at 10 mM final concentration) and the reaction was stopped by addition of Na_2CO_3 solution to a final concentration of 0.5 M. The liberated *p*-nitrophenol was determined by E_{410} measurements. One unit of enzyme activity liberates 1 μmol of *p*-nitrophenol/min of incubation under the above conditions, and specific activities are expressed in milliunits/mg of protein. Results are given as mean $\pm$ S.E.M. Numbers of experiments are given in parentheses where relevant.

Results

Growth and development

Myxamoebae of *D. discoideum* Ax-2 grow in axenic medium with a mean generation time of approx. 8 h and when harvested in the exponential phase of growth and placed on solid substratum the cells go through a well-defined sequence of morphological stages in 24 ± 1 h (Watts & Ashworth, 1970). In bacterial suspensions myxamoebae of *D. discoideum* Ax-2 grow with a mean generation time of approx. 4 h and take 27 ± 1 h to differentiate into fruiting bodies on solid substratum. In agitated buffer suspension the *D. discoideum* Ax-2 cells develop aggregation competence in about 8 h (Malchow *et al.*, 1972; Lee, 1972) and if the cells are placed on Millipore filters after shaking in buffer for 6–24 h they develop complete fruiting bodies in 22 ± 1 h instead of the usual 27 ± 1 h. Thus for the first 5–6 h of development it does not appear to matter whether cells are on solid substratum or in suspension, but for further development it seems that the cells need normal cell-cell contacts on a moist solid substratum in contact with air.

Throughout exponential growth, the cellular protein remains relatively constant at 9.1 ± 0.2 (10) mg/ 10^8 cells for axenically grown cells and at 6.01 ± 0.15 (14) mg/ 10^8 cells for myxamoebae grown in bacterial suspension. However, during development under all conditions there is a considerable loss of protein (Fig. 1).

Rates of glycosidase accumulation

Figs. 2–5 show the changing β -N-acetylglucosaminidase and α -glycosidase activities during growth

and differentiation under a variety of conditions. Similar information has been obtained for a number of other glycosidases but is not presented in detail, since these two enzymes appear to be representative. From these data the absolute rate at which cells accumulate or lose assayable enzyme activity can be calculated as follows.

In a population of growing or developing slime-mould cells, any small increase in assayable enzyme activity (dE) occurring in a small interval of time (dt) is proportional to the total number of cells in that population (N) during the time interval dt , that is:

$$\frac{dE}{dt} \propto N \quad (1)$$

and

$$\frac{dE}{dt} = k_a N \quad (2)$$

where k_a is the zero-order rate constant for accumulation of assayable enzyme activity (accumulation rate constant), expressed as enzyme units $\cdot$ time $^{-1}$ $\cdot$ cell number $^{-1}$.

Rearrangement of eqn. (2) and integration between the time limits t_1 and t_2 gives the formula:

$$E_{t_2} - E_{t_1} = k_a \int_{t_1}^{t_2} N dt \quad (3)$$

and since during development N is constant,

$$E_{t_2} - E_{t_1} = k_a N (t_2 - t_1) \quad (4)$$

During exponential growth, k_a was calculated as a routine from eqn. (3). $E_{t_2} - E_{t_1}$ is the amount of total enzyme activity (cellular plus excreted enzyme) accumulated by the culture in the interval of time

Fig. 1. Changes in the total cellular protein content during the development of *D. discoideum* Ax-2

Protein content was measured in axenically grown myxamoebae differentiating on Millipore filters (Δ) [graph taken from Hames & Ashworth (1974)]; in bacterially grown myxamoebae differentiating on Millipore filters ($\square$); and in bacterially grown myxamoebae differentiating in buffer suspension ($\bullet$). For details see the Materials and Methods section.

Fig. 2. Accumulation of β -N-acetylglucosaminidase activity during growth of *D. discoideum* Ax-2 myxamoebae

The myxamoebae were grown (a) in a suspension of *E. coli* B/r and (b) in axenic medium. Measurements were made of cell number (Δ), cellular enzyme activity ($\blacksquare$) (nmol/min per ml of culture), extracellular enzyme activity ($\square$) (nmol/min per ml of culture), and specific activity in myxamoebae ($\circ$). For full experimental details see the Materials and Methods section.

Fig. 3. Accumulation of α -glucosidase activity during growth of *D. discoideum* Ax-2 myxamoebae

The conditions for this Figure and the symbols are the same as in Fig. 2.

Fig. 4. Accumulation of β -N-acetylglucosaminidase activity during development of *D. discoideum* Ax-2

Cells were initially grown (a) in a suspension of *E. coli* B/r and (b) in axenic medium. Measurements were made of enzyme activity in cells developed on filters (▲) and in suspension (△), of extracellular enzyme activity from cells developed on filters (■) and in suspension (□), and of specific activity in cells developed on filters (●) and in suspension (○). For full experimental details see the Materials and Methods section.

$t_2 - t_1$. The definite integral $\int_{t_1}^{t_2} N dt$, of the growing myxamoebal population during the time-interval, can be graphically measured from the surface area circumscribed by the growth curve. Graphs of total enzyme activity are not shown in Figs. 2-5 but in the case of bacterially grown cells, they are essentially

the same as the plot of total cellular enzyme activity/ml of culture against time, because enzyme secretion is negligible during exponential growth.

During development, eqn. (3) can be used to calculate k_a as described above. However, since the accumulation of enzyme activity was generally linear with time over the first few hours of development, and

Fig. 5. Accumulation of α -glucosidase activity during development of *D. discoideum* Ax-2

The conditions for this Figure and the symbols are the same as in Fig. 4(a).

the cell number was relatively constant, eqn. (4) was used as a routine. Table 1 compares the accumulation rate constants of several *D. discoideum* Ax-2 glycosidases during growth and development under a variety of conditions. Except for α -mannosidase, k_a was relatively constant for the glycosidases throughout exponential growth. The accumulation constant for α -mannosidase activity decreased during exponential growth in both axenic and bacterial media by approx. 50%.

The specific activity of β -N-acetylglucosaminidase throughout growth of myxamoebae of strain NC-4 (Fig. 6) and the cell-generation times are approximately the same as for myxamoebae of strain Ax-2 growing in bacterial suspension (Fig. 2a). This indicates that the k_a values for the accumulation of total β -N-acetylglucosaminidase activity for the two strains, when grown on bacteria, are approximately the same.

Effect of cyclic AMP on enzyme accumulation

Cyclic AMP is known to affect the rates of accumulation (synthesis) and secretion of a number of glycosidases in a variety of systems (Varmus *et al.*, 1970; Van Wijk & Konijn, 1971; Weissmann *et al.*, 1971; Rosness *et al.*, 1971). Thus since cyclic AMP is synthesized and secreted during axenic growth and differentiation of *D. discoideum* Ax-2

Fig. 6. β -N-Acetylglucosaminidase activity during growth of myxamoebae of strain NC-4 on the surface of agar in association with *Klebsiella aerogenes*

The results of two experiments are shown. The arrow indicates the time at which all bacteria were consumed.

cells (Malkinson & Ashworth, 1972, 1973) at the same time as the synthesis and secretion of glycosidases, it was thought possible that a relationship might exist between these two phenomena. However, altered concentrations of exogenous cyclic AMP have insignificant effects on the accumulation and secretion of β -N-acetylglucosaminidase activity.

Discussion

The results in this paper, together with those of several other workers (Loomis, 1969a, 1970; Coston & Loomis, 1969; Quance & Ashworth, 1972; Ashworth & Quance, 1972) show that in *D. discoideum* the specific activity of five glycosidases changes when cells stop exponential growth. However, only with α -mannosidase and β -glucosidase do these changes in enzyme activity correspond to a change in the actual rate of accumulation of total enzyme activity (units/h per cell); β -N-acetylglucosaminidase, α -glucosidase and β -galactosidase activities have an essentially constant rate of accumulation throughout exponential growth and the first few hours of development (Table 1). During exponential growth, although a single cell accumulates enzyme activity, it also accumulates total protein, such that in terms of specific activity the enzyme remains at a relatively low

Table 1. Accumulation rate constants (k_a) of glycosidase activities during growth and development of *D. discoideum* Ax-2

For full experimental details see the Materials and Methods, and Results, sections. Results are given as means $\pm$ S.E.M. Numbers of experiments are given in parentheses.

Enzyme	Accumulation rate constants for total enzyme activity, k_a (units/h per 10^8 cells)				
	Bacterially grown myxamoebae			Axenically grown myxamoebae	
	Exponential growth*	Development		Exponential growth*	Development on solid substratum
		On solid substratum	In suspension		
β -N-Acetylglucosaminidase	70.3 $\pm$ 2.8 (2)	92.5 $\pm$ 3.5 (2)	74.2 $\pm$ 3.0 (4)	275.5 $\pm$ 4.1 (4)	260 $\pm$ 10 (2)
α -Mannosidase	4.5 $\pm$ 0.3 (2)	8.3 $\pm$ 0.5 (2)	6.8 $\pm$ 0.5 (3)	84.3 $\pm$ 4.1 (4)	—
α -Glucosidase	1.60 $\pm$ 0.08 (2)	1.4 $\pm$ 0.2 (2)	1.42 $\pm$ 0.04 (3)	2.75 $\pm$ 0.12 (4)	—
β -Glucosidase	14.7 $\pm$ 0.4 (2)	-4.7 $\pm$ 0.5 (2)	-6.0 $\pm$ 0.2 (2)	3.71 $\pm$ 0.16 (4)	—
β -Galactosidase	1.71 $\pm$ 0.05 (2)	1.35 $\pm$ 0.15 (2)	1.30 $\pm$ 0.05 (3)	4.06 $\pm$ 0.11 (4)	—

* Except for α -mannosidase, k_a measurements were made for glycosidases in cultures of myxamoebae growing exponentially on bacteria from 1.5×10^6 cells/ml to 1.2×10^7 cells/ml, and on axenic medium from 7.5×10^5 cells/ml to 6×10^6 cells/ml. For α -mannosidase, k_a measurements were made between 8×10^5 cells/ml and 3.5×10^6 cells/ml for bacterially grown myxamoebae cultures and between 7.5×10^5 cells/ml and 2.5×10^6 cells/ml for axenically grown myxamoebae cultures.

value. Total enzyme activity per cell also remains low because of cell division. During differentiation a single cell accumulates enzyme activity at the same rate as during growth (Table 1), but total protein actually falls (Fig. 1) so that in terms of specific activity a rapid increase occurs. Total enzyme activity per cell also increases because the cell does not divide. After several hours of development the rate of enzyme accumulation falls to zero and the rise in specific activity reaches a plateau. In some cases the enzyme activity per cell then declines, owing to a combination of enzyme excretion and degradation.

The changes in α -mannosidase activities can be explained partly by changes in the rate of total protein turnover and cell division as described above for β -N-acetylglucosaminidase, but for α -mannosidase, changes in rate of enzyme accumulation play a greater role; there is a gradual decline in rate during growth and a sudden increase in rate at the beginning of differentiation (Table 1) corresponding to the sudden increase in specific activity.

β -Glucosidase activities seem to be regulated in the same way as β -N-acetylglucosaminidase during growth, but on the initiation of development there is a rapid fall in enzyme activity owing to enzyme excretion and degradation.

Whatever the mechanisms for the regulation of glycosidase activities may be, the results in this paper indicate that the synthesis of these enzymes is independently regulated because the ratio of their accumulation rate constants changes markedly under different conditions of growth. For example, the k_a values for α -glucosidase, β -galactosidase, α -mannosidase, β -glucosidase and β -N-acetylglucosaminidase are in the approximate proportions 1:1:3:9:44 when myxamoebae are grown in

bacterial suspension, as compared with the proportions 1:1.5:31:1.4:100 when myxamoebae are grown axenically. Also, the times during growth or differentiation at which the accumulation of the various glycosidases is terminated is different for each enzyme (Figs. 2-5; Loomis, 1969a, 1970; Coston & Loomis, 1969; Ashworth & Quance, 1972; Quance & Ashworth, 1972).

We thank the New Zealand Ministry of Agriculture and Fisheries, The Science Research Council (U.K.) and the University of Leicester for financial support. We also thank Miss J. Johnson and Mrs. J. Kwasniak for technical assistance.

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