Intracellular Protein Breakdown in Thermophilic and Mesophilic Fungi

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1. The rate of protein breakdown was determined on growing and non-growing cultures of thermophilic and mesophilic fungi. 2. In growing cells protein breakdown was negligible. 3. In non-growing cells the breakdown rate of total protein varied between 5.2%/h and 6.7%/h. These values were found to be dependent on both the temperature of the protein breakdown assay and the temperature of growth of the organism. 4. The rate of breakdown of soluble protein in thermophilic fungi was 9–15%/h whereas the rate in mesophilic fungi for the soluble protein fraction was only 4%/h.

Two general theories have been advanced to explain the ability of thermophilic micro-organisms to grow at high temperatures: (a) that the cellular components of thermophiles are more thermostable than the corresponding components in mesophiles (Amelunxen & Lins, 1968; Farrell & Campbell, 1969; Pace & Campbell, 1967; Farrell & Rose, 1967); (b) that thermophilic organisms survive at high temperatures because they have the ability to replace heat-damaged intracellular material rapidly. The rapid-turnover theory was initially proposed by Allen (1953) and supported by the studies carried out by Bubela & Holdsworth (1966). More recently, however, it has been shown that the rate of protein turnover in thermophilic bacteria is the same as that found in mesophilic bacteria (Epstein & Grossowicz, 1969; Coulta & Sundaram, 1971). Growing cells generally exhibit a turnover rate of less than 3%/h (Mandelstam, 1958), whereas much faster rates of turnover are found in non-growing cells (3–7%/h) (Epstein & Grossowicz, 1969; Mandelstam, 1958) and sporulating cells (18–22%/h) (Spudich & Kornberg, 1968; Bernlohr, 1972). The present paper reports the results of protein breakdown in growing and non-growing cells from thermophilic and mesophilic fungi.

Materials and Methods

Materials

All inorganic chemicals were analytical-reagent grade. The [U-¹⁴C]phenylalanine and U-¹⁴C-labelled protein hydrolysate (from *Chlorella vulgaris* cells) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Yeast extract and casamino acids (vitamin-free) were from Difco Laboratories, Detroit, Mich., U.S.A. Casein hydrolysate was from British Drug Houses Ltd., Poole, Dorset, U.K., and peptone was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Other chemicals were

obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Cells

The thermophilic fungi *Penicillium duponti* (A.T.C.C. 26229) and *Malbranchea pulchella* var. *sulfurea* were obtained from Dr. G. M. Gaucher, University of Calgary, Canada. *Mucor miehei* (N.R.R.L. 3169), a thermophile, and *Penicillium notatum* (N.R.R.L. 832), *Penicillium chrysogenum* (N.R.R.L. 811) and *Aspergillus oryzae* (N.R.R.L. 695), all mesophiles, were obtained from the Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill., U.S.A. The thermophile *Aspergillus fumigatus* was a gift from Dr. H. O. W. Eggins, University of Aston, Birmingham, U.K.

Culture conditions

All strains of thermophilic and mesophilic fungi used were propagated on yeast-glucose agar as described previously (Miller & Shepherd, 1972). Submerged cultures were prepared by growing thermophiles at 45°C and mesophiles at 28°C on a New Brunswick Gyrotory Shaker (250 rev./min) in 500 ml Erlenmeyer flasks containing 250 ml of autoclaved modified Czapek–Dox medium (1% glucose) (Gaucher & Shepherd, 1968). After 24 h, samples of each culture (25 ml) were used to inoculate flasks containing 250 ml of temperature-equilibrated Czapek–Dox medium, in which 0.1% peptone replaced the 0.3% yeast extract (CDP medium). The cells were grown on the gyrotary shaker for a further 18 h.

Methods

Protein breakdown in non-growing cells. Samples of each fungus (20 ml), prepared as described above, were diluted with 80 ml of temperature-equilibrated

CDP medium containing 0.1 μ mol of [U- 14 C]-phenylalanine (10Ci/mol)/100ml. These cultures were incubated with shaking, in 250ml Erlenmeyer flasks, for a further 10h at their respective growth temperatures, at which time the labelling of the protein had reached a steady state (Fig. 1). The cells were then harvested by filtration, washed and resuspended in 100ml of CDP medium devoid of glucose and containing an excess of unlabelled phenylalanine (1g/100ml). Cultures were incubated on the gyrotary shaker at their respective growth temperatures and 10ml samples were removed hourly for 6h. Each sample was immediately filtered and the cells were then washed and resuspended in 10ml of 0.01M-Tris-HCl buffer, pH7.0 (0°C). The suspensions were then treated for 1min in an ultrasonic disintegrator. Samples (1ml) of the resulting suspensions were used for total protein estimation and 2.5ml of each suspension was added to 1ml of 17.5% (w/v) trichloroacetic acid to precipitate protein. The remaining 6.5ml of each suspension was diluted to 10ml in the Tris-HCl buffer and centrifuged at 20000g for 15min. The resulting supernatants were centrifuged at 105000g for 90min at 4°C in a Beckman L.2-65B ultracentrifuge. A 1ml portion of each supernatant was used for estimating the soluble protein concentration and 2.5ml was added to 1ml of 17.7% trichloroacetic acid to precipitate soluble protein. Each trichloroacetic acid-precipitated sample was boiled for 30min and the protein was collected on a membrane (0.45 μ m; Millipore Corp., Bedford, Mass., U.S.A.) and washed with 5% trichloroacetic acid. The filtered material was dried and the radioactivity was determined.

To study the effect of temperature on protein breakdown, *P. duponti* was grown at 40°, 45° and 50°C and *P. notatum* was grown at 23°, 28° and 33°C. The rate of protein breakdown was determined at each growth temperature; e.g., the breakdown rate of *P. duponti* mycelia grown at 40°C was determined at 40°C. In another experiment *P. duponti* and *P. notatum* were grown at the temperatures given above, but the protein breakdown assay was performed in each case at 45°C for *P. duponti* and 28°C for *P. notatum*. The total protein concentration and protein radioactivity were determined at hourly intervals as described above.

Protein breakdown in growing cells. *P. duponti* and *P. notatum* were cultivated in 250ml of Czapek-Dox medium as described. After 24h the cells were harvested by filtration and resuspended in 300ml of Czapek-Dox medium devoid of yeast extract, containing 0.001% casein hydrolysate and 0.25 μ Ci of U- 14 C-labelled protein hydrolysate (57mCi/mg-atom of C)/100ml. The cultures, in 500ml Erlenmeyer flasks, were incubated on the gyrotary shaker for 90min at 45° and 28°C respectively. The cultures were then divided into three equal parts and an excess of

unlabelled amino acids (casamino acids; 10%, w/v, final concentration) was added. Each flask was incubated, with shaking, for 3h at one of three different temperatures: 5°, 28° and 45°C for *P. notatum* cells and 28°, 45° and 65°C for *P. duponti* cells. Every 30min, samples (10ml) were removed from each flask and these were assayed for total protein concentration and protein radioactivity as described above.

Determination of protein radioactivity. Radioactivity was counted in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The efficiency of counting 14 C radioactivity with the use of a toluene-based scintillant was 69%.

Determination of protein. Protein concentration was measured as described by Eggstein & Kreutz (1967).

Measurement of protein breakdown. The rate of protein breakdown in non-growing cells was determined by measuring the decrease in c.p.m./mg of protein for total and soluble protein of each fungus. The results were expressed as a percentage decrease in c.p.m./mg of protein of the first sample taken in each assay. For Figs. 2-4 each point represents the average value of at least two experiments.

Results

In order to study protein turnover in these fungi, resting mycelial suspensions had to be obtained under conditions closely resembling the growth conditions. The results in Table 1 show that resting mycelial suspensions can be produced by removing glucose from the growth medium, CDP; removal of NaNO₃ was not sufficient to prevent growth. Fig. 1 shows (a) that protein synthesis occurs in non-growing mycelial suspensions of both *P. duponti* and *P. notatum* while there is no increase in the size of the total protein pool and (b) that the labelling of protein reaches a steady

Table 1. Growth of *P. duponti* and *P. notatum* in liquid media

P. duponti and *P. notatum* were grown for 48h in modified Czapek-Dox medium at 45° and 28°C respectively as described in the Materials and Methods section. The cells were harvested, washed and resuspended in sterilized glass-distilled water. Samples of these suspensions (5ml) were used to inoculate 100ml of media indicated in the table and each solution was grown for a further 24h before harvesting and determination of the increase in dry weight.

Medium	Increase in dry weight (g/l)	
	<i>P. duponti</i>	<i>P. notatum</i>
Czapek-Dox	6.8	6.1
CDP	1.2	3.5
CDP minus NaNO ₃	1.2	3.0
CDP minus glucose	0	0

Fig. 1. Incorporation of $[U-^{14}C]$ phenylalanine into trichloroacetic acid-precipitated protein of non-growing cells of *P. duponti* and *P. notatum*.

P. duponti (a) and *P. notatum* (b) were grown, as described in the Materials and Methods section, at 45° and 28°C respectively. Samples of each culture (20 ml) were harvested, washed and resuspended in 100 ml of CDP medium minus glucose (non-growth medium) containing 8 μ Ci of $[U-^{14}C]$ -phenylalanine (80 Ci/mol). The suspensions were then incubated at their respective growth temperatures and 10 ml samples were removed each hour. Samples were assayed for trichloroacetic acid-precipitated protein radioactivity and protein concentration as described in the Materials and Methods section. ●, Protein radioactivity; △, protein concentration.

Fig. 2. Protein breakdown in growing *P. notatum* cells.

P. notatum was grown in CDP medium at 28°C and protein was made radioactive with $U-^{14}C$ -labelled protein hydrolysate as described in the Materials and Methods section. The protein breakdown assay was carried out at 28°C. ●, Protein radioactivity; ▲, protein concentration.

state after 6 h. If, after 6 h of incubation, glucose was added to the non-growing mycelial suspension, the mycelium began to grow again.

Protein breakdown in growing fungi

Breakdown of cellular protein labelled with $U-^{14}C$ -labelled protein hydrolysate was measured in growing cultures of *P. duponti* and *P. notatum*. Fig. 2 shows a typical experiment for protein breakdown in a growing culture of *P. notatum* incubated at 28°C; there was no change in the radioactivity of the protein fraction after incubation for 3 h in the CDP medium. After the 3 h incubation, however, the total protein had increased almost twofold. Similar results were obtained for growing cultures of *P. duponti* incubated at 45°C. There was no change in the protein radioactivity and no increase in protein concentration when this experiment was carried out at incubation temperatures of 28° and 65°C for *P. duponti* and 5° and 45°C for *P. notatum*.

Protein breakdown in non-growing fungi

The maximum rate of protein breakdown for *P. duponti* (6.7%/h) was obtained at 45°C, whereas for *P. notatum* a maximum rate of protein breakdown of 6.5%/h was found at 28°C, i.e. their optimum growth temperatures (Miller & Shepherd, 1972). *P. notatum* mycelia, grown at temperatures of 23°, 28° and 33°C, showed protein breakdown rates at 28°C of 5.3%, 6.5% and 8.9%/h respectively (Fig. 3b). *P. duponti* mycelia, grown at temperatures of 40°, 45° and

Fig. 3. Effect of temperature on protein breakdown in non-growing *P. duponti* and *P. notatum* cells

P. duponti (a) was grown at 45°C and *P. notatum* (b) was grown at 28°C in CDP medium. Protein was made radioactive with [U-¹⁴C]phenylalanine as described in the Materials and Methods section. The protein breakdown assay for non-growing *P. duponti* cells was carried out at 28°C (Δ), 45°C (○), 50°C (▲) and 65°C (●). The protein breakdown assay for non-growing *P. notatum* cells was carried out at 5°C (●), 23°C (▲), 28°C (○) and 45°C (Δ).

Fig. 4. Breakdown of total and soluble protein in non-growing *P. duponti* and *P. notatum* cells

P. duponti (a) was grown at 45°C and *P. notatum* (b) was grown at 28°C in CDP medium. Protein was made radioactive with [U-¹⁴C]phenylalanine as described in the Materials and Methods section. The protein breakdown assays were carried out at 45°C for *P. duponti* and 78°C for *P. notatum*. ○, Total protein; ●, soluble protein.

50°C, showed protein breakdown rates at 45°C of 6.1%, 6.7% and 9.6%/h respectively (Fig. 3a).

Rate of breakdown of total and soluble protein

The rate of breakdown of total and soluble protein from *P. duponti* and *P. notatum* was measured as shown in Fig. 4. These data show that breakdown of soluble protein was more rapid than breakdown of total protein in *P. duponti* (9.2%/h, cf. 6.7%/h). The rates of breakdown of total and soluble protein from three other thermophiles and two other mesophiles are

Table 2. Rate of total and soluble protein breakdown in thermophilic and mesophilic fungi

The method for the preparation of the soluble protein fraction and measurement of protein breakdown rates were as described in the text. Values are expressed as %/h and are the averages of duplicate experiments.

Organism (resting cells)	Growth temp. (°C)	Total protein breakdown (%/h)	Soluble protein breakdown (%/h)
<i>Penicillium duponti</i>	45	6.7	9.2
<i>Aspergillus fumigatus</i>	45	6.0	11.7
<i>Mucor miehei</i>	45	5.9	13.1
<i>Malbranchea pulchella</i> var. <i>sulfurea</i>	45	6.7	15.2
<i>Penicillium chrysogenum</i>	28	5.2	3.9
<i>Aspergillus oryzae</i>	28	6.2	4.0

shown in Table 2. The rate of breakdown of total protein varied between 5.2% and 6.7%/h for all of the fungi examined. A marked difference, however, was found in the rates of breakdown of soluble protein. In the thermophiles the rate of breakdown of this protein fraction was about twice the rate of total protein breakdown, whereas in the mesophiles the rate of breakdown of soluble protein was much lower than the total protein breakdown rate.

Discussion

Although protein turnover has been studied extensively in bacterial systems (Mandelstam, 1960; Pine, 1972) and mammalian systems (Ciba Foundation, 1973), protein turnover has not been studied in fungi. The study of protein turnover in fungi presented several difficulties. No defined medium could be produced in which the thermophilic fungi would grow, and the method for determining fungal protein radioactivity had to be modified from the methods previously used with bacterial systems.

Protein turnover in non-growing bacteria has been studied in a number of media by removing any of a number of components such as nitrogen (Schlessinger & Ben-Hamida, 1966; Epstein & Grossowicz, 1969), glucose or phosphate (Willettts, 1967a), or by using specific mutants (Mandelstam, 1958). We found that the modified CDP medium provided good growth for both fungi used in this study, and non-growth conditions were produced when glucose was removed from this medium (Table 1). The removal of nitrogen or phosphate from the CDP medium was not sufficient to produce non-growth conditions.

In bacterial studies the radioactivity of the cells was used as an index of the radioactivity of the total protein (Epstein & Grossowicz, 1969). This method cannot be applied to fungal systems, since large variations in radioactivity were found when small samples were withdrawn. Therefore in these studies larger samples were taken (10ml) and made completely homogeneous by sonication.

In this study, although only protein breakdown was measured, it is assumed that similar results would have been obtained if protein turnover, i.e. breakdown and resynthesis, had been measured. Our justifications for this assumption are (a) that protein synthesis is occurring in non-growing fungal cell suspensions (Fig. 1) and (b) that Mandelstam (1958) has shown in non-growing bacterial suspensions that the rate of breakdown of protein is equal to the rate of synthesis of protein. Fig. 2 shows that protein breakdown in growing fungi is negligible, and this is supported by studies with bacterial systems, where a rate of less than 1%/h was obtained (Willettts, 1967b). In non-growing fungi the rate of breakdown was found to vary between 5.2% and 6.7%/h (Table 2). The rate of protein turnover in non-growing bacterial suspensions of thermophiles and mesophiles was found to vary between 5% and 7%/h (Mandelstam, 1958; Urbā, 1959; Willettts, 1967a), and was also found to be influenced by the temperature of the assay (Epstein & Grossowicz, 1969). Our results show that the rate of protein breakdown for *P. duponti* and *P. notatum* increased as the growth temperature was increased, but that the maximum rate of protein breakdown always occurred when the assay was performed at the optimum temperature of growth (Fig. 3).

A marked difference was found in the rate of breakdown of soluble protein from the thermophilic fungi compared with the rate of breakdown of soluble protein from the mesophilic fungi (Table 2). In thermophiles, the rate of breakdown of soluble protein was about twice the rate of the total protein breakdown, whereas in mesophiles the rate of breakdown of soluble protein was considerably lower than the total protein breakdown rate. The soluble protein in the fungi we examined accounted for only 10–20% of the total protein, which explains why the soluble protein breakdown rate did not greatly affect the breakdown rate of total protein.

The observation that, in the thermophilic fungi, the rate of soluble protein breakdown is higher than the rate of total protein breakdown suggests that, in order to survive at high temperatures, these fungi have developed the capacity to rapidly resynthesize the labile soluble proteins. The insoluble proteins are associated with other cellular components and this may protect them from degradation. In contrast with the thermophilic fungi, the lower rate of breakdown of soluble protein in mesophilic fungi

suggests that these proteins are relatively stable at their temperature of synthesis. The difference between the rate of breakdown of the total and soluble protein fractions of mesophilic fungi may not be significant.

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