Effect of Metabolic Conditions on Protein Turnover in Yeast

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1. In yeast growing on ethanol a turnover rate of up to 2%/h was measured. As much as 80% of the protein was subject to turnover, and no marked heterogeneity in the rate of degradation of protein was observed. When the yeast grew on glucose, the protein was degraded at a lower rate (0.5–1%/h). 2. Starvation for a nitrogen source increased the rate of protein degradation severalfold, whereas deprivation of phosphate had only a marginal effect (30% increase). Removal of glucose from a medium containing 50 mM-phosphate did not cause marked changes in the rate of protein degradation. In contrast, when the media were low in phosphate (0.1 mM) removal of glucose increased the rate of turnover 2–4-fold. 3. Protein degradation proceeded unimpaired when the intracellular concentration of ATP decreased from 4 to 1 mM, but stopped completely when it decreased below 0.3 mM.

Materials and Methods

Organisms and growth procedures

The following *Saccharomyces cerevisiae* strains were used: CJM 13 (provided as 1714-24 by Professor D. C. Hawthorne, Washington University, Seattle, WA, U.S.A. CJM 13arg–, a derivative from CJM 13 that requires arginine for growth; and CJM 22 [strain MC-6A from Henry et al. (1975)] provided by Professor D. G. Fraenkel, Harvard Medical School, Boston, MA, U.S.A. The yeasts were grown with aeration at 30°C in a minimal-salts medium (Olson & Johnston, 1949) by using NaCl (0.25 g/litre) instead of the original medium citrate and adjusting the initial pH of the medium to 5.5 by the addition of 5m-NaOH (medium A). In some experiments a medium without phosphate (Chung & Nickerson, 1954) was used (medium B). As carbon and energy sources, 2% glucose or 2% ethanol was added. Media devoid of a nitrogen source were prepared by replacing the ammonium salts with the corresponding potassium salts.

Labelling of yeast protein and measurement of protein degradation

A culture of yeast in the exponential phase of growth (0.8–1.5 mg of fresh yeast/ml) was incubated with a labelled amino acid (L-[U-14C]leucine; 300 mCi/mmol at 5 μM final concn., unless otherwise indicated) for one or two generations. (The generation times were: on glucose, 2h for strain CJM 13 and 4h for strain CJM 22; on ethanol, 6h for strain CJM 13.) The yeast was collected by centrifugation.
(2 min/3700 g), washed twice with prewarmed medium, and resuspended (0.2–0.6 mg of fresh yeast/ml) in fresh medium as indicated in each particular experiment. At appropriate times 0.1 ml portions of the yeast suspension were diluted with 0.9 ml of cold water and mixed with 2 ml of 15% (w/v) trichloroacetic acid. After heating for 15 min at 90°C, the suspension was filtered through a glass-fibre filter (Whatman GF/C, 2.5 cm diam.), washed twice with 2 ml of 5% trichloroacetic acid and once with 2 ml of a mixture of ethanol/ether (1:1, v/v). The filters were transferred to a scintillation vial with 2.5 ml of a scintillation fluid that contained 7.5 g of PPO (2,5-diphenyloxazole) and 0.75 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in 1 l of toluene and 0.5 litre of Triton X-100. Radioactivity was counted in a Nuclear-Chicago mark-II liquid-scintillation counter.

The release of trichloroacetic acid-soluble material was measured by adding 0.8 ml of the yeast suspension to 0.2 ml of 50% trichloroacetic acid. After at least 3 h the suspension was filtered and 0.2 ml of the filtrate were used for counting radioactivity by adding 2.5 ml of the scintillation fluid described above. The results were calculated as the percentage of the initially present trichloroacetic acid-insoluble radioactivity that appears in the soluble fraction (after correction for the acid-soluble radioactivity present at zero time).

In all experiments reported, each value is the mean of results from duplicate or triplicate samples.

Hydrolysis of the trichloroacetic acid-insoluble fraction and electrophoresis of the hydrolysate

Portions of the labelled yeast suspension were treated with hot trichloroacetic acid as described in the previous section. The sediments were hydrolysed with 2 ml of 6M HCl in sealed ampoules for 20 h at 110°C. The hydrolysates were concentrated to dryness in a rotary evaporator at 44°C, and the residues suspended in 0.15 ml of distilled water.

Portions of the concentrated hydrolysates were submitted to electrophoresis for 1 h at 30 V/cm on Whatman 3MM paper, with formic acid/acetic acid/water (1:3:36, by vol.) as solvent. The paper strips were dried and cut into 2 cm fragments; each piece was placed in a scintillation vial and radioactivity was counted as described above. A leucine standard was run in parallel and its position in the electrophoretogram located with ninhydrin.

Determination of ATP

ATP was extracted by incubation of the yeast (0.4–2 mg) into 2M perchloric acid (final vol. 1.2 ml) for 5 min in the cold. After 5 min centrifugation at 4000 g, 0.8 ml of the supernatant was neutralized with 0.8 ml of a solution containing 1.8 M-KOH and 0.4 M-KHCO₃. After 5 min in the cold the suspension was centrifuged, and ATP was measured in the supernatant by measuring the luminescence produced by luciferin–luciferase as described by Wilson et al. (1976).

Chemicals

Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Enzymes and nucleotides were from Boehringer, Mannheim, Germany, or from Sigma, St. Louis, MO, U.S.A. Firefly-lantern extract was obtained from Sigma. All other reagents were of analytical grade.

Results and Discussion

Adequacy of the method used for measuring the rate of degradation of protein

To follow the rate of degradation of the proteins in a cell, different methods have been used (Pine, 1972; Goldberg & Dice, 1974; Ballard, 1977). The most common one has been to add a labelled amino acid to the medium for a short time and measure the decrease of radioactivity in the proteins as a function of time. If the rate of degradation is low, the measurements of radioactivity in protein give very imprecise results, and the release of labelled trichloroacetic acid-soluble material is measured instead. However, this procedure would lead to erroneous results if the released amino acid is actively metabolized to volatile or trichloroacetic acid-insoluble products such as CO₂ or polysaccharides.

To test the adequacy of this method for yeast, protein was labelled with [14C]leucine and radioactivity was measured in parallel in the trichloroacetic acid-insoluble fraction and in the soluble material. Since preliminary experiments showed that the rate of protein turnover varied in different strains, several strains of S. cerevisiae were tested. As Table I shows, under the conditions used the radioactivity appearing in the soluble fraction is equivalent to the radioactivity lost from the protein. However, in nitrogen starvation, leucine can be metabolized to some extent, releasing CO₂, as shown by the fact that at least 30% of the radioactivity disappearing from the insoluble fraction can be trapped with alkali. It is therefore necessary to check in each experiment whether after extensive degradation all the radioactivity lost from the protein is conserved in the trichloroacetic acid-soluble fraction. Essentially all the label in the trichloroacetic acid-insoluble fraction was associated with leucine (Fig. 1), thus indicating that leucine is not significantly metabolized to trichloroacetic acid-insoluble material different from proteins.

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Table 1. Correlation between radioactivity lost from protein and radioactivity appearing in the trichloroacetic acid-soluble fraction

Yeast growing on medium A with 2% glucose were labelled with [U-14C]leucine as described in the Materials and Methods section. The yeasts were resuspended in fresh medium containing 2% glucose, 10 mM-unlabelled leucine, and, in the case of strain CJM 13 arg-, 0.1 mM-arginine. At the times indicated, portions were withdrawn, and the radioactivity present in the trichloroacetic acid-soluble and in the trichloroacetic acid-insoluble fractions was determined as described in the Materials and Methods section. The radioactivity incorporated in the protein at zero time was 3.0 x 10^4 c.p.m. in the sample corresponding to strain CJM 22, 1.6 x 10^4 c.p.m. for strain CJM 13 and 1.4 x 10^4 c.p.m. for strain CJM 13 arg-.

<table>
<thead>
<tr>
<th>S. cerevisiae strain</th>
<th>Fraction</th>
<th>Time (h)</th>
<th>Radioactivity recovered (%)</th>
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<tbody>
<tr>
<td>CJM 22</td>
<td>Soluble</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Insoluble</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td></td>
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<td>96</td>
</tr>
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<td>CJM 13</td>
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<tr>
<td></td>
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<td>96</td>
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Fig. 1. Distribution of radioactivity in a hydrolysate of the trichloroacetic acid-insoluble fraction

S. cerevisiae CJM 13 growing on medium A with 2% glucose was labelled for 2.5h with [U-14C]leucine as described in the Materials and Methods section. The yeast was then resuspended in medium A without nitrogen; portions were taken at zero time (○) and after 9h (●) and 31h (△) and the trichloroacetic acid-insoluble fractions hydrolysed and submitted to electrophoresis as described in the Materials and Methods section. The arrow indicates the origin, and the shaded area the position of leucine as detected with ninhydrin in a standard run in parallel.

Re-utilization of labelled amino acids for the synthesis of new protein (amino acid recycling) would lead to an underestimation of the rate of protein degradation. Davies & Humphrey (1978) have determined leucine recycling in Schizosaccharomyces pombe, and have shown that 4 or 5h after the withdrawal of labelled leucine the error introduced by recycling is small. However, measurements performed before this time were greatly affected by the re-utilization of leucine. We have tested the effect of adding different concentrations of unlabelled leucine on the apparent degradation rate of yeast protein; the results are shown in Fig. 2. The release of trichloroacetic acid-soluble radioactivity is seen to be slow if no unlabelled leucine is added to the medium. The apparent degradation rate increases when leucine is present, and the results are similar in the range 1-25 mM-leucine. Leucine up to 25 mM does not affect the yeast growth rate and therefore is not likely to have marked secondary physiological effects on the yeast. In the present work, cold 10 mM-leucine has been routinely added to the medium where degradation was measured.

Care should be taken when choosing a labelled amino acid to measure protein degradation in yeast. When leucine is used the intracellular soluble fraction contains only 1% of the radioactivity incorporated in the protein; by contrast, if arginine is used, after 3h labelling as much radioactivity is found in the soluble fraction as in the protein, and this intracellular arginine does not exchange readily with the arginine in the medium. This is probably due to the trapping of arginine in the vacuole (Wiemken & Dürr, 1974), and makes this amino acid unsuitable for the measurement of protein turnover.

Since different proteins have differing degradation rates, labelling them during a short time would tend to label preferentially proteins with the highest turnover rate. When the yeast is actively growing, however, the whole set of proteins is being synthesized and labelled, and after one or two generation times the bias introduced by short-lived proteins is minor.
**Effect of the carbon source**

It has been reported (Hansen et al., 1977) that the activities of proteinases in yeast are dependent on the carbon source used for growth, glucose acting as a repressor. Protein degradation was therefore compared in yeast growing on ethanol and on glucose. The results are presented in Fig. 3. When the yeast was pre-grown in glucose, the degradation rate was 2-3-fold higher in ethanol than in glucose, the difference being already noticeable after 1h incubation in the new medium. When the yeast was pre-grown in ethanol, the degradation was similar for the first hour in ethanol and in glucose, but afterwards the degradation rate decreased in the glucose culture.

These results suggest a correlation between the activity of proteinases found in the yeast and the protein-degradation rate. However, it does not seem that there is a direct proportion between protein turnover and proteinase activity, since these activities can show up to 10-fold differences (Hansen et al., 1977), and a change in the carbon source produces at most a 3-fold variation in the degradation rate. The results indicate also that, as a whole, the proteins of yeast grown on glucose are slightly more stable than those of yeast grown on ethanol.

It should be stressed that no marked heterogeneity in the rate of degradation of yeast proteins has been observed. In ethanol-grown yeast at least 50% of the proteins have a half-life of around 40h. After this time, degradation slows down, but that could be due to the depletion of the carbon source and cessation of growth. In fact, in conditions of very slow growth up to 80% of the initial protein is degraded after 7 days (Fig. 4). The biphasic aspect of the degradation plot could be due to the marked decrease in growth rate observed after 50h or to a heterogeneity in the rate of degradation of the different yeast proteins. The situation is, in any case, very different from that...
reported for *Escherichia coli* (Nath & Koch, 1970), where about 5% of the proteins show a half-life of 1 h, no proteins of intermediary stability are found, and where the slow-decaying (about 0.4%/h) component observed probably does not even represent degradation of intracellular protein to amino acids.

**Effect of the absence of nitrogen or phosphate**

Protein breakdown has been reported to increase when *E. coli* is deprived of essential nutrients (Goldberg *et al.*, 1975). In yeast the apparent degradation rate reaches 2%/h in a medium without nitrogen source (Fig. 5). This is, however, a minimal estimate, since in the absence of unlabelled leucine the reutilization of the labelled amino acid liberated during protein degradation is quite high, as shown in the control with a nitrogen source and without added leucine. On the other hand, if leucine is added to the medium as usual, the yeast is able to use this amino acid as a nitrogen source, and no nitrogen deprivation takes place.

Protein degradation has also been measured in a mutant auxotroph for arginine, by using either a complete medium with NH$_4^+$, arginine and leucine or a medium devoid of arginine and NH$_4^+$ but still containing leucine. A 5-fold increase in the rate of protein degradation was observed in the absence of arginine. The results reported are in agreement with the preceding ones and indicate that protein degradation is very sensitive to a nitrogen deficit.

In the absence of phosphate, a 30% increase in the rate of protein breakdown has been consistently observed, but this increase is much smaller than that found in *E. coli*. Probably on account of the large amounts of polyphosphates stored by yeast (Harold, 1966; Indge, 1968), no complete deprivation of phosphate occurs even when it is omitted from the medium.

**Effect of the absence of carbon source**

When yeast is grown on a mineral medium with 50mM-phosphate, only slight differences are observed on removal of glucose (Fig. 6). In contrast, when a medium with only 0.1mM-phosphate is used, a marked increase in the degradation rate occurs in the absence of glucose (Fig. 6).

When the labelling and degradation are performed in a medium containing ethanol, the removal of the carbon source does not increase the degradation rate even at a low concentration of phosphate, an observation that shows that in yeast the absence of a carbon source does not always result in increased
Fig. 6. Effect of glucose removal on the degradation rate of yeast protein at different phosphate concentrations in the medium

The protein of *S. cerevisiae* growing in medium A (50 mM-phosphate) with 2% glucose (○, ●) or in medium B (0.1 mM-phosphate) with 2% glucose (Δ, ▲) was labelled with [U-14C]leucine for a generation time as described in the Materials and Methods section. Portions of the yeasts were resuspended in fresh medium containing 10 mM-leucine and with (○, Δ) or without (●, ▲) 2% glucose. At the times indicated portions were withdrawn and the radioactivity present in the trichloroacetic-acid soluble fraction was determined as described in the Materials and Methods section. (a) Strain CJM 13; (b) strain CJM 22.

protein degradation; the nature of the carbon source used for growth plays a role, and so does the concentration of phosphate in the medium. That could point to the energy state of the cell as a factor controlling protein turnover; this will be discussed in the section below.

**Effect of the energy state of the yeast**

Although the reaction catalysed by proteinases does not require ATP, the protein-degradation process appears to be energy-dependent (Hershko & Tomkins, 1971; Goldberg *et al*., 1975, 1976). In sporulating yeast, protein degradation has been reported to be inhibited by CN⁻, F⁻, valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone (Betz & Weiser, 1976), but no measurements have been made of the range of ATP concentrations allowing degradation to occur.

Yeast can be depleted of ATP by addition of arsenate or 2,4-dinitrophenol in the absence of a carbon source. When the ATP concentration decreases below 0.3 mM, protein degradation stops completely (results not shown). When yeast growing on glucose is resuspended in a medium without carbon source, the ATP concentration decreases from 4 to 1 mM (Fig. 7); this decrease, however, does not prevent an increase in the rate of protein degradation (cf. Figs. 6 and 7). Since after ethanol removal the ATP concentration decreased very slowly (Fig. 7) and no increase in protein degradation rate took place, it could be suggested that an abrupt decrease in the concentration of ATP increases the rate of protein degradation. It is clear, however, that other factors play a role in the control of protein turnover, since there is not always a correlation between changes in the ATP concentration and modification of the protein-degradation rates (cf. Figs. 6 and 7). Further work is needed to pinpoint other control elements in the degradation process.

**Final remarks**

The first data on protein degradation in yeast obtained by Halvorson (1958a,b) indicated that in growing *S. cerevisiae* the degradation rate was negligible (less than 0.1%/h). More recently Waldron *et al.* (1977) stated also that protein from growing *S. cerevisiae* was stable. These results contrast with the reports of Betz (1976), Bakalkin *et al.* (1976) and with the present results. The indirect method used by Halvorson (1958a,b) could be the reason why he did not observe protein degradation. As for Waldron *et al.* (1977), the method they used would not allow the detection of a low rate of turnover, since under their conditions some re-utilization of leucine would occur, and they measured decrease of radioactivity in protein for only 6 h.
It should also be stressed that turnover of protein appears to vary among species and even among strains. For *S. cerevisiae* growing on glucose we have found turnover rates between 0.5 and 1 %/h; Betz (1976) reports values of 1 %/h and Bakalkin *et al.* (1976) of 3 %/h. This last value, however, may be too high, since degradation was measured only for 1 h and, as seen in Fig. 6, the degradation rate tends to be higher in the first hour than thereafter. Sims *et al.* (1974) did not observe any protein degradation in growing *Candida utilis*, although it is possible that their method (measure of decrease of radioactivity in protein for only 3 h) would have overlooked a low turnover. On the other hand, Davies & Humphrey (1978) report a degradation rate of about 4 %/h in growing *Schizosaccharomyces pombe*.

The depletion of nutrients has been shown to increase the turnover rate, although the values obtained are again dependent on strain and species; Betz (1976) reported a degradation rate of 2 %/h in

*Saccharomyces* starved for essential amino acids, we observed degradation rates of up to 3 % in the absence of glucose, and Davies & Humphrey (1978) showed a degradation rate as high as 10 % in *Schizosaccharomyces* suspended in phosphate buffer.

It can therefore be concluded that yeast protein is always subject to degradation, although the protein turnover can be overlooked if insensitive methods are used. The turnover rates in turn can vary greatly depending on strains, species and metabolic conditions.

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References


Hershko, A. & Tomkins, G. M. (1971) J. Biol. Chem. 246, 710–714


