# INHIBITION OF PROTEIN SYNTHESIS STIMULATES INTRACELLULAR PROTEIN DEGRADATION IN GROWING YEAST CELLS

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Received July 6,1976

## SUMMARY

In exponentially growing cells of Saccharomyces cerevisiae, cycloheximide stimulated intracellular protein degradation to the same extent as did starvation for required amino acids. By using inhibitors of macromolecular synthesis and temperature-sensitive mutants defective in different steps of RNA and protein synthesis it could be demonstrated, that this stimulation of protein degradation was directly related to the inhibition of protein synthesis per se, but not connected to the cessation of ribosomal RNA synthesis or to the inhibition of cell growth.

## INTRODUCTION

During initiation of yeast sporulation the degradation of vegetative cell proteins is severely inhibited by cycloheximide (1,2). In contrast, during the later stages of sporulation as well as in stationary yeast cells intracellular protein degradation is insensitive to the drug (1,2). This discrepancy had been explained by the assumption that during the initial stages of sporulation cycloheximide prevents the synthesis of a protein or a proteinase required for the adaptation of the intracellular proteolytic apparatus to increased rates of protein degradation (1). When the effect of cycloheximide on protein degradation was investigated with exponentially growing cells, I surprisingly found that under these conditions the drug caused the same stimulation of intracellular proteolysis as did starvation for required amino acids. In the

present paper, the relationship between stimulation of intracellular proteolysis and cessation of macromolecular synthesis in growing cells has been investigated.

## MATERIALS AND METHODS

Saccharomyces cerevisiae, strain A 364 A (a $ad_1$ $ad_2$ $ur_1$ $ty_1$ $hi_7$ $ly_2$ $ga_1$), was kindly provided by Dr. L.H. Hartwell, University of Washington, Seattle, Washington. The temperature-sensitive mutants $ts$ 136 ($rna_1$), $ts$ 166 ($rna_6$), $ts$ 187 ($prt_1$), $ts$ 241 ($prt_2$) and $ts$ 368 ($rna_2$) originally isolated from strain A 364 A by Dr. L.H. Hartwell (3,4,5,6,7) were obtained from the Yeast Genetic Stock Center, Donner Laboratory, Berkeley, California. The cells were grown in the following medium: 2% (w/v) glucose, 0.67% (w/v) nitrogen base without amino acids (Difco Lab., Detroit, Michigan), 0.001% (w/v) adenine, 0.001% (w/v) uracil, 2 mM tyrosine, histidine and lysine, each, and 50 mM sodium phosphate, pH 5.7. For starvation experiments, either uracil or the amino acids were omitted. All experiments were performed with growing cells at a cell density of about 2 x $10^7$ cells per ml.

Protein degradation was measured as described previously (1) by following the release of trichloroacetic acid-soluble material from cells grown in the presence of L-[4,5-$^3$H]leucine (1 µCi per ml culture) after transfer of the cells to the respective medium containing 2 mM L-leucine as a chase. The only modification was that transfer of the labelled cells to the new medium was accomplished by collecting the cells from 10 ml aliquots of the culture on Millipore GSWP filters (0.22 µm), washing the cells twice with 10 ml prewarmed resuspension medium and suspending the cells in 10 ml of the new medium. The results were calculated as the percentage of the initially present trichloroacetic acid-insoluble radioactivity that becomes acid-soluble in the course of the experiment after correction for the acid-soluble radioactivity present at zero-time. Differences in quenching were monitored by internal standardization.

RNA synthesis was measured by the incorporation of [8-$^{14}$C] adenine (54.2 mCi/mmol: Amersham-Buchler, Braunschweig, GFR) similar to the method developed by Hartwell (3). In these experiments, the washed cells were transferred to medium containing 0.25 µCi [8-$^{14}$C] adenine per ml. At the times indicated, 1 ml samples of the culture were removed and mixed with 1 ml ice-cold 10% (w/v) trichloroacetic acid. The precipitates were collected on Whatman GFC filters and five times washed with 10 ml cold 5% (w/v) trichloroacetic acid containing 2 mM adenine. The filters were dried and counted in a toluene-based scintillation mixture.

## RESULTS

In the experiment shown in Fig. 1, protein degradation in strain A 364 A was estimated during growth on complete medium and during amino acid and uracil starvation both in the absence and presence of cycloheximide. After transfer of the growing cells to

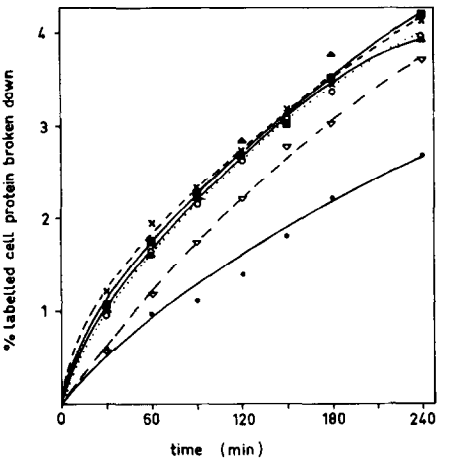


Fig. 1: Effect of amino acid or uracil starvation and of cycloheximide on protein degradation in strain A 364 A. After labelling of the cells with L-[$^3$H]leucine during growth at 30°C and transfer to fresh media containing unlabelled L-leucine, protein degradation was determined as detailed under Materials and Methods. ●——● complete medium; x - - x complete medium + 20 µg/ml cycloheximide; ▲——▲ medium without amino acids; o...o medium without amino acids + 20 µg/ml cycloheximide; ▽- -▽ medium without uracil; ■——■ medium without uracil + 20 µg/ml cycloheximide.

fresh complete medium, a basal rate of protein degradation of about 1% per hour was observed. Starvation for the required amino acids lysine, histidine and tyrosine resulted in an about twofold enhancement of protein degradation. The same stimulation of protein degradation could be achieved by the addition of cycloheximide to cells growing on complete medium. Since during amino acid starvation the intracellular pools of the required amino acids become reduced, whereas inhibition of protein synthesis by cycloheximide usually increases the amino acid pool sizes (8,9), it is evident that the rate of protein degradation cannot simply be regulated by the intracellular amino acid or aminoacyl-tRNA levels as had been proposed (9,10). When the cells were starved for the required base uracil, a similar but less pronounced stimulation of protein degradation com-

pared to amino acid-starved cells was observed. Cycloheximide treatment of amino acid- or uracil-starved cells caused no additional enhancement of protein degradation compared to the stimulation of proteolysis observed in unstarved cycloheximide-poisoned cells. This indicates that the stimulation of protein degradation obtained with all these different treatments may be exerted by a rather similar or even the same mechanism. Unfortunately, both amino acid and uracil starvation and cycloheximide treatment cause rather pleiotropic effects in growing cells, as for example inhibition of macromolecular synthesis and growth arrest (11,12). Since in bacteria the regulation of ribosomal RNA synthesis and of protein degradation has been attributed to a common genetic control by the *rel*$^+$ locus (13,14,15), in the following experiments the relationship between protein degradation and RNA, DNA and protein synthesis has been studied by the use of respective inhibitors and of temperature-sensitive mutants defective in different steps of RNA and protein synthesis.

As has been established by other authors (11,12) and is shown for strain A 364 A in Tab. 1, both amino acid and uracil starvation

Tab. 1: Effect of amino acid and uracil starvation and of cycloheximide on RNA accumulation in strain A 364 A. RNA accumulation was measured after transfer of the growing cells to fresh media containing [$^{14}$C]adenine as detailed under Materials and Methods.

| resuspension medium | [$^{14}$C]adenine incorporated in 180 min (counts min$^{-1}$ ml culture$^{-1}$) |
|---|---|
| complete medium | 533 300 |
| medium without amino acids | 62 800 |
| medium without uracil | 34 800 |
| complete medium + 20 µg/ml cycloheximide | 56 600 |

as well as cycloheximide treatment caused not only a marked inhibition of protein synthesis (data not shown), but also a cessation of net RNA accumulation. Since ribosomal RNA accounts for at least 80% of the RNA of the cell, the cessation of RNA accumulation is mainly due to the inhibition of ribosomal RNA synthesis. The starvation- or cycloheximide-induced stimulation of protein degradation was, however, not related to the inhibition of ribosomal RNA synthesis nor to the cessation of cell growth, since neither inhibition of RNA accumulation by 8-hydroxyquinoline (16) nor inhibition of DNA synthesis by hydroxyurea (17) showed any effect on the basal rate of protein degradation (Fig. 2).

The lack of any correlation between cessation of ribosomal RNA synthesis and stimulation of intracellular proteolysis could in ad-

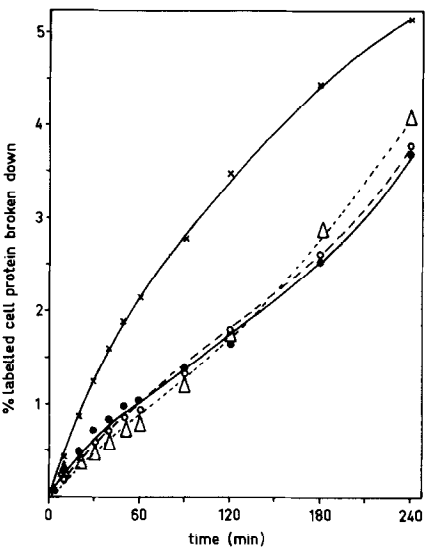


Fig. 2: Effect of inhibitors of macromolecular synthesis on protein degradation in strain A 364 A. The experimental procedures were identical to those of Fig. 1. ●——● complete medium; x——x complete medium + 20 µg/ml cycloheximide; Δ---Δ complete medium + 50 µg/ml 8-hydroxyquinoline; o - - o complete medium + 75 mM hydroxyurea.

dition be confirmed by using temperature-sensitive mutants defective in the synthesis or accumulation of either ribosomal RNA (ts 166 and ts 368; 3,5) or total cytoplasmic RNA (ts 136; 4). The parent strain of these mutants, strain A 364 A, showed according to the $Q_{10}$ of most biological reactions an about 2-fold higher rate of protein degradation at 36°C compared to that at 23°C (Fig. 3A). The mutants ts 166 and ts 368, which are both defective in ribosomal RNA synthesis (5), exhibited a similar stimulation of protein degradation at the restrictive temperature of 36°C as found in strain A 364 A at least during the first 2 to 3 hours after the shift. Following this initial period, the rate of protein degradation decreased because of yet unknown reasons (data not shown). In any case, the similar initial rates of protein degradation at 36°C in both the mutants and strain A 364 A again suggested that stimulation of protein degradation is not mediated by the cessation of ribosomal RNA synthesis. In mutant ts 136, a shift of the cells to 36°C reproducibly resulted in an enhanced rate of protein degradation compared to that of the parent strain A 364 A. This latter mutant is defective in the production or transport of all types of cytoplasmic RNA and has been shown to exhibit a decay of polyribosomes to monoribosomes and a concomitant decrease in the rate of protein synthesis at the restrictive temperature (4). It therefore appeared possible that the enhanced stimulation of proteolysis in ts 136 at 36°C was due to the inhibition of protein synthesis under these conditions.

In order to prove for this latter interpretation, the same temperature-shift experiments were performed with conditional mutants defective in different steps of protein synthesis. As is shown in Fig. 3 B, mutant ts 187, which is defective in the initiation of protein synthesis and exhibits a very rapid inhibition of protein accumulation at the restrictive temperature (7), exhibited an almost

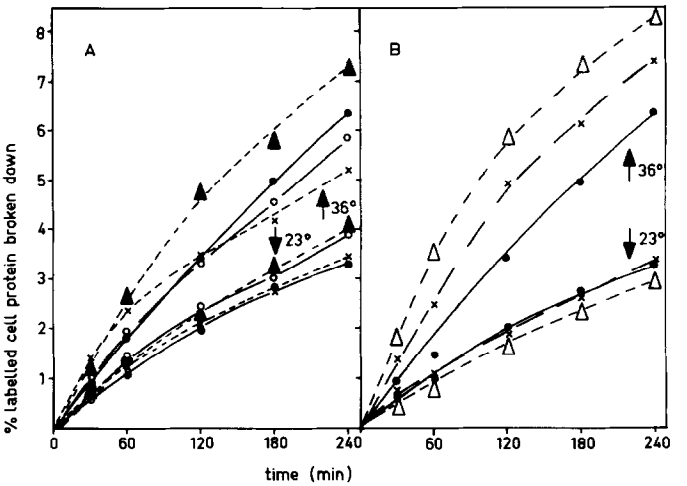


Fig. 3: Protein degradation in strain A 364 A and mutants ts 136, ts 166, ts 187, ts 241 and ts 368 at 23°C and 36°C. The cells of strain A 364 A and of the temperature-sensitive mutants were labelled during growth at 23°C with L-[$^3$H]leucine as described under Materials and Methods. After transfer to fresh complete medium containing unlabelled L-leucine, aliquots were cultured at 23°C and 36°C, respectively. Protein degradation was followed as detailed under Materials and Methods. A: ●——● A 364 A; ▲---▲ ts 136; x - - x ts 166; o——o ts 368. B: ●——● A 364 A; △- -△ ts 187; x——x ts 241.

twofold higher stimulation of protein degradation in the first hours after the shift to 36°C than strain A 364 A. An increased, but less pronounced stimulation of protein degradation at 36°C was also observed with mutant ts 241, which is defective in polypeptide chain elongation (6). From these results it is concluded that any inhibition of protein synthesis itself, regardless whether exerted at the level of initiation (ts 187) or elongation (ts 241, cycloheximide, amino acid starvation; 6,7), stimulates the rate of intracellular proteolysis.

## DISCUSSION

The first conclusion to be drawn from this investigation is that the inhibition of protein synthesis stimulates protein degrad-

ation in growing yeast cells. In accord with my findings, an accelerated loss of several enzyme activities and of labelled protein from cycloheximide-treated yeast cultures has been reported by Sims et al. (18). At present, it is not known whether this stimulation of proteolysis is due to an accelerated degradation of all cellular proteins or to an enhanced degradation of a specific class of proteins. In any case, the stimulatory effect of protein synthesis inhibitors on intracellular proteolysis should be considered in all experiments in which these drugs are used with growing yeast, i.e. for example in studies on enzyme stability and on mitochondrial protein synthesis.

Second, the experiments with various inhibitors of macromolecular synthesis and with temperature-sensitive mutants defective in different steps of RNA and protein synthesis revealed that any inhibition of translation is effective in stimulating intracellular proteolysis. Both arrest of polypeptide chain initiation (ts 187) and elongation (ts 241; cycloheximide treatment; amino acid starvation) have been shown to enhance the rate of protein degradation. Since initiation, amino-acyl-tRNA binding and translocation all involve GTP hydrolysis, one may speculate that in inhibited cells the accumulation and possibly an alternate fate of GTP may constitute the primary trigger for the stimulation of proteolysis.

Third, from this and from previous studies on protein degradation in sporulating yeast (1,2) it emerges that in Saccharomyces cerevisiae at least two different mechanisms must be involved in the maximal stimulation of intracellular proteolysis, the one being activated, the other being inhibited by cycloheximide. As has been shown in this paper, a stimulation of protein degradation, i.e. an activation of the preexisting proteolytic apparatus of the cell, was observed when the cells were starved for required compounds

(amino acids or uracil) or treated with cycloheximide in a medium containing an excess of glucose to be used for ATP production. When, however, yeast is starved in the absence of glucose, as is for example the case after transfer to sporulation medium or during the transition from the exponential to the stationary growth phase, the cells not only stimulate their preexisting proteolytic apparatus, but in addition adapt to further enhanced rates of protein degradation by the induced synthesis of required proteinases (1,19). As already mentioned, in such adapting cells protein degradation is inhibited by cycloheximide (1,2). The response of the yeast cell to starvation for a required amino acid or other nitrogen sources thus seems to be tightly controlled by the availability of the fermentable sugar glucose. In fact, both the synthesis of the various yeast proteinases and of their specific protein inhibitors have been shown to be under the control of glucose catabolite repression (19,20; R. J. Hansen, unpublished experiments).

Acknowledgment I thank Professor H. Holzer for his interest and support during the course of this work and Dr. B.A. Hemmings for a critical reading of the manuscript. The assistance of Mrs. L. Kötschau and Miss S. Zehender during the preparation of the manuscript is gratefully acknowledged. This work had been supported by the Deutsche Forschungsgemeinschaft, SFB 46.

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