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ASPARAGINASE II OF *SACCHAROMYCES CEREVISIAE*

INACTIVATION DURING THE TRANSITION TO STATIONARY PHASE

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Summary

Asparaginase II (L-asparagine amidohydrolase, EC 3.5.1.1) activity of cells from stationary phase cultures of *Saccharomyces cerevisiae* is very low. When these cells are inoculated into minimal medium, asparaginase II specific activity rises rapidly and reaches a maximum after 9—10 h. During the next 2.5—3 h, a rapid decrease in asparaginase II specific activity occurs. The enzyme does not appear to be secreted into the medium or to be reabsorbed into the cell. Addition of protease inhibitors at the time of maximum activity partially or totally prevents the loss of asparaginase II. L-1-Tosylamide-2-phenylethyl chloromethyl ketone decreases the rate of loss. The sulfhydryl reagents *p*-hydroxymercuribenzoate and iodoacetamide inhibit the loss of asparaginase II. However, addition of EDTA causes a further increase in activity. This increase is due to de novo protein synthesis. The effect of EDTA can be reversed by the addition of Zn^{2+}. The most likely explanation for the rapid loss of asparaginase II is proteolytic degradation by a Zn^{2+}-dependent, thiol protease or peptidase.

Introduction

An important feature of yeasts is the existence of cell-bound enzymes that are outside the permeability barrier of the plasma membrane and thus readily

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Abbreviations: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

accessible to their substrates or to changes in the external environment [1,2]. The most thoroughly studied of these enzymes is invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26), which exists in both intracellular and extracellular forms [3]. Similarly, there are two distinct forms of asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) in *Saccharomyces cerevisiae* [4—7].

L-Asparaginase I is a constitutive enzyme that deamidates only intracellular L-asparagine [4—7]. In contrast, asparaginase II is a glycoprotein that can deamidate either L- or D-asparagine when it is external to the cell [5—7]. In fact, the V for D-asparagine is slightly higher than that for the L-isomer [7]. The location and substrate preference of asparaginase II can be exploited to distinguish unambiguously between the two asparaginase activities. In addition, the fact that asparaginase II can be assayed with washed, intact cells makes it possible to answer questions that could not be considered if cell-free extracts were required to detect activity.

Asparaginase II has been extensively characterized by its discoverers [5—7]. It is an extremely stable phosphomannan protein that retains complete activity between pH 3.5 and 10.5 [7]. This enzyme can be strongly derepressed by starving cells for nitrogen in the presence of an energy source [5].

We have shown that cells growing in minimal medium with an abundant nitrogen source (NH_4^+) have high asparaginase II activity in early exponential phase [8]. Rapid loss of activity occurs as cultures approach the end of exponential phase but before the growth rate decreases appreciably. Supplementing cultures just before the loss begins with metabolites such as D-glucose, NH_4^+, L-arginine, L-proline, L-glutamate, L-aspartate, or D-asparagine does not affect the loss of activity. However, addition of L-asparagine or L-glutamine increases the time required for the loss to occur from 2.5—3 h to 7 h. If protein synthesis is blocked during the hour before the loss begins, no loss occurs. In contrast, inhibition of protein synthesis at the peak of activity or later results in considerable loss of activity [8].

In the present paper we have examined the events governing this rapid loss of activity. There are at least three possible means by which loss of extracellular activity might occur. 1. The cells might secrete the enzyme into the medium. 2. The enzyme might be taken back into the cell. 3. The enzyme might be inactivated in situ. This could occur by proteolysis, demannosylation, phosphorylation or dephosphorylation, complexing with a specific inhibitor, or some other process.

Although turnover and inactivation of internal enzymes, in response the changing nutritional conditions, have been investigated extensively in yeast and other microorganisms [9,10], we are not aware of any studies that have examined the rapid loss of an external enzyme in yeast. Finkelstein and Strausberg [11], and independently, Ciejek and Thorner [12], described experiments that suggest that the yeast mating pheromone α-factor is degraded by surface-bound endopeptidase(s). In an analogous manner, our data suggest that proteolytic degradation is responsible for the rapid loss of asparaginase II activity.

Materials and Methods

The following were obtained from Sigma Chemical Co. (St. Louis, MO): D-asparagine, EDTA, TPCK, TLCK, *p*-hydroxymercuribenzoate, iodoacetamide, pepstatin, chloroquine, PMSF, *o*-phenanthroline, 8-hydroxyquinonline, 2,2′,2″-tripyridine, NaN_3, con A-agarose, cycloheximide, glutamate dehydrogenase. All other chemicals were reagent grade.

Yeast strains. The *S. cerevisiae* wild type strain S288C was obtained from the Yeast Genetics Stock Center, University of California, Berkeley, CA. The carboxypeptidase S mutant *cps* 31-1A (*leu cbxY*$^-$ *cbxS*$^-$) was obtained from Dr. D.H. Wolf [13,14].

Media and growth of cells. Media were prepared as described previously [4]. Cells were grown at 28 ± 1°C on rotary shakers in minimal medium with vitamins [4]. Growth was monitored as described previously [8].

Genetic methods. Yeast crosses and segregational analyses have been described [4].

Enzyme assays. Asparaginase II was assayed as described previously [8]. In brief, cells were collected on Millipore filters, washed with 20 mM potassium phosphate buffer (pH 7.0), and resuspended in this buffer. D-Asparagine to a final concentration of 10 mM was added to initiate the reaction. The reaction was stopped by boiling for 6 min and the amount of NH_4^+ released was determined by secondary coupling with glutamate dehydrogenase. Protein concentrations were determined as described previously [8]. 1 unit of enzyme activity produces 1 nmol of NH_4^+/min.

Preparation of inhibitors. All inhibitors were added from concentrated stock solutions. When necessary, inhibitors were dissolved in 95% ethanol or dimethylsulfoxide. Controls to which solvent alone was added showed no detectable effect of the solvent upon loss of asparaginase II.

Toluene treatment of cells. Cells were harvested, washed three times with 20 mM potassium phosphate buffer (pH 7.0), and resuspended in this buffer. One-tenth volume of toluene or buffer was added. The suspensions were then mixed vigorously for 1 min before being placed in a 30°C water bath for 5 min. The cells were assayed for asparaginase II activity immediately after being removed from the water bath.

Results

Asparaginase II activity in rapidly growing cells

The time course of the rise and fall of asparaginase II in exponential phase cultures is shown in Fig. 1. Maximum activity (arrow A) occurs when the culture density is about $1.7 \cdot 10^7$ cells/ml. In all the inhibitor studies described in this paper, the inhibitors were added at this time. Effects of the inhibitors were determined by measuring activity at the time indicated by arrow B, except when the effects were measured as a function of time. The culture density at B is about $3.8 \cdot 10^7$ cells/ml.

We have examined a number of possibilities to elucidate the mechanism responsible for loss of asparaginase II activity at this specific point in the growth cycle. Sakamoto et al. [15] have recovered asparaginase from the medi-

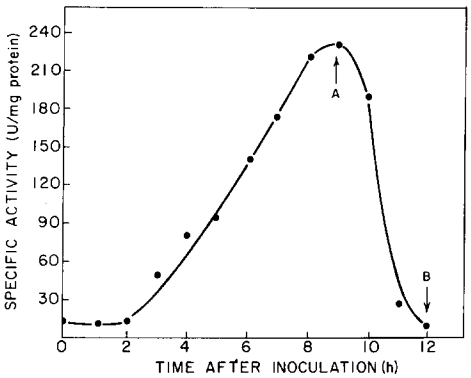

Fig. 1. Time course of asparaginase II specific activity accumulation and loss in cells growing in minimal medium. Stationary phase cells were seeded into fresh medium to an initial cell density of $9 \cdot 10^5$ cells/ml. Growth conditions are described in the text. Samples were removed at the indicated times and immediately assayed for asparaginase II activity. The time indicated by the arrow A represents the time when inhibitors were added in subsequent experiments. Arrow B indicates the time when the cultures were assayed to determine the effect of the inhibitor.

um of stationary phase cultures of the yeast *Candida utilis*. In light of this, we have tried several different methods to identify asparaginase II activity in the filtrate of cultures grown to a density of $3.8 \cdot 10^7$ cells/ml. Because the assay for asparaginase II measures the production of NH_4^+, the filtrates were either dialyzed exhaustively or desalted with Sephadex G-25 to remove NH_4^+.

In one set of experiments, the dialysate was concentrated by evaporation and assayed. No asparaginase II activity was found. The assay conditions allow detection of enzyme activity as low as 0.15 units.

In another set of experiments, ice-cold ethanol was added to the dialysate to a final concentration of 50%; asparaginase II is not soluble at this ethanol concentration [7]. The redissolved precipitate was assayed, but no activity was found.

In a third set of experiments, the knowledge that asparaginase II binds to concanavalin A was exploited [7]. The dialysate was slowly stirred at 4°C for 30 min with con A-agarose beads. The preparation was then eluted with 5% α-methyl-D-mannoside [7]. No activity was recovered from the beads stirred with the culture filtrate. Under these same conditions, all of the asparaginase II in a cell-free extract containing a very high level of activity was adsorbed to the con A-agarose (data not shown). Using the same elution conditions, more than 50% of the activity was recovered from the beads reacted with the extract (data not shown). These three sets of experiments strongly suggest that the rapid loss is not due to secretion of active enzyme into the medium.

Because asparaginase II is assayed by measuring the deamidation of substrate that is external to the cell, it is possible that the loss of activity is caused by reabsorption of the enzyme into the cell where it is no longer accessible to external substrate. If this is the case, and the enzyme remains in an active form after being sequestered, then cells from a culture of $3.8 \cdot 10^7$ cells/ml should be able to deamidate D-asparagine after being made permeable with toluene. As shown in Table I, there is virtually no difference in activity between permea-

TABLE I

ASPARAGINASE II ACTIVITY IN CELLS MADE PERMEABLE WITH TOLUENE

Cells were harvested, washed, and resuspended in buffer. Toluene treatment of cells is described in Materials and Methods. A volume of buffer equal to the volume of toluene was added to the untreated cells. The cells then were assayed for asparaginase II. Values represent the mean ± the standard error of the mean of triplicate samples.

Culture density	Asparaginase II specific activity (units/mg protein)	
	—Toluene	+Toluene
$1.7 \cdot 10^7$ cells/ml	218 ± 1	198 ± 6
$3.8 \cdot 10^7$ cells/ml	26 ± 0.3	26 ± 0.3

bilized and nonpermeabilized cells from either exponential or transition phase cultures. The trivial explanation that toluene denatures asparaginase II is ruled out by the exponential phase activities. These results indicate that the enzyme is not reabsorbed by the cell, although these data do not entirely eliminate the possibility that it is sequestered in an inactive form.

Effects of protease inhibitors

Given that asparaginase II activity could not be located either in the culture fluid or inside the cell, we were left with the possibility that the enzyme is inactivated in situ, perhaps by proteolysis. Accordingly, a variety of protease inhibitors was added to cultures at the time of maximum activity to attempt to block possible degradation of the enzyme. Asparaginase II was measured 2.5—3 h after addition of inhibitor (Table II).

Well known serine protease inhibitors such as PMSF and *p*-toluenesulfonyl chloride could not prevent the loss. Chloroquine, pepstatin, and TLCK have been shown to inhibit the extracellular proteolysis of the mating pheromone

TABLE II

EFFECT OF PROTEASE INHIBITORS ON LOSS OF ASPARAGINASE II ACTIVITY

Inhibitors were added to cultures at the time of maximum activity. Cells were harvested, washed and assayed for asparaginase II activity 3 h later. (N.R.) Not reported. *p*-Hydroxymercuribenzoate, *p*-OHMB. Effectiveness refers to the protection of asparaginase II or α-factor activity

Inhibitor (final concentration)	Inhibitor Specificity (ref.)	Effectiveness	
		Asparaginase II	α-factor *
PMSF (1 mM)	serine proteases [16]	—	—
p-Toluenesulfonyl chloride (1 mM)	serine proteases [17]	—	—
Chloroquine (1 mM)	lysosomal proteases [18]	—	+
Pepstatin (1 mM)	yeast protease A [19]	—	+
Cu^{2+} (0.5 mM)	yeast protease B [20]	—	N.R.
TLCK (1 mM)	trypsin [21]	—	+
TPCK (1 mM)	chymotrypsin [22]	+	—
p-OHMB (5 mM)	thiol proteases [23]	++	N.R.
Iodoacetamide (5 mM)	thiol proteases [23]	++	N.R.
EDTA (5 mM)	metalloproteases [23]	++	—

* Data from Refs. 11 and 12.

α-factor [11,12], but they were ineffective in our system. In contrast to the findings with α-factor [11,12], TPCK and metal chelators were effective in preventing loss of asparaginase II activity.

When the chymotrypsin inhibitor TPCK was tested at a concentration of 1 mM, about 67% of the activity was retained after 3 h. This represents a 4—6-fold increase over control levels. Unfortunately, higher concentrations of TPCK could not be tested because of solubility problems. The time course of loss of activity after addition of TPCK is shown in Fig. 2. Addition of TPCK at the time of maximum activity increased the time required for loss of asparaginase II from 3 to 7 h.

The sulfhydryl reagents *p*-hydroxymercuribenzoate and iodoacetamide were found to be highly effective at preventing the inactivation. The dose-response curve for *p*-hydroxymercuribenzoate (Fig. 3) shows that inactivation is inhibited (80%) by concentrations greater than $3 \cdot 10^{-3}$ mM. The kinetics of the loss after addition of *p*-hydroxymercuribenzoate could not be determined, because this compound is highly toxic to the cells, and the cultures stopped growing shortly after its addition. Asparaginase II activity itself is not inhibited by *p*-hydroxymercuribenzoate [5,7].

Effect of divalent cation chelators

We observed two different effects after addition of chelating agents (Table III). Chelators that can penetrate the cell membrane (e.g. 8-hydroxyquinoline, *o*-phenanthroline, 2,2′,2″-tripyridine and NaN_3) completely inhibited the inactivation of asparaginase II. 8-Hydroxyquinoline and *o*-phenanthroline have been shown to inhibit RNA synthesis in yeast, probably by chelating the Zn^{2+} cofactor of RNA polymerase [24,25].

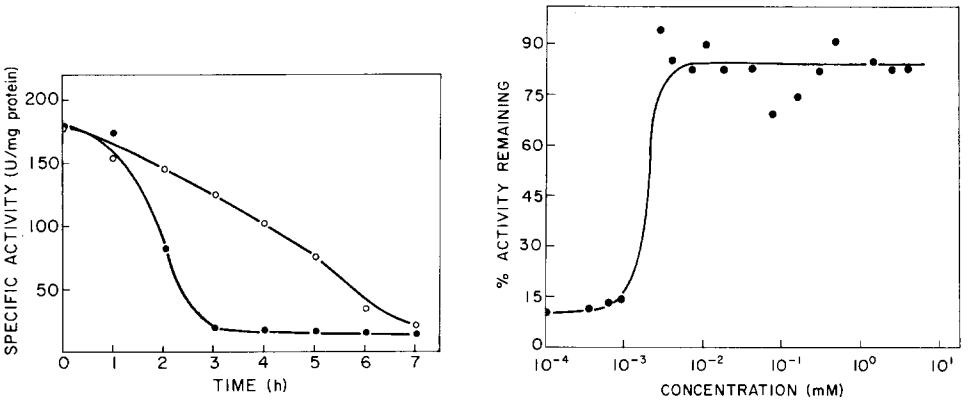

Fig. 2. Effect of 1 mM TPCK on the kinetics of loss of asparaginase II from rapidly growing cells. Cells were grown in minimal medium to a density of $1.7 \cdot 10^7$ cells/ml, at which time TPCK was added. The control culture continued to grow in the original medium. Samples of the cultures were removed at the times indicated and assayed immediately for asparaginase II. Control culture (●———●); TPCK (○———○).

Fig. 3. Effect of increasing concentration of *p*-hydroxymercuribenzoate on loss of activity of asparaginase II from rapidly growing cells. Cells were grown in minimal medium to a density of $1.7 \cdot 10^7$ cells/ml. The cultures then were supplemented with the indicated final concentrations of *p*-hydroxymercuribenzoate and incubated for 3 h. At this time, cells were harvested and assayed immediately for asparaginase II.

TABLE III

EFFECT OF DIVALENT CATION CHELATORS ON LOSS OF ARPARAGINASE II ACTIVITY

Chelators were added to cultures at the time of maximum activity (specific activity 210 units/mg protein). Cells were assayed after 2.5—3 h incubation with inhibitor. Values represent the mean ± the S.E. of the mean of triplicate samples.

Chelator (final concentration)	Asparaginase II specific activity (units/mg protein)
none	11 ± 1
o-phenanthroline (5 mM)	226 ± 4
8-hydroxyquinoline (2.8 mM)	216 ± 4
2,2′,2″-tripyridine (1 mM)	228 ± 4
NaN_3 (10 mM)	221 ± 4
EDTA (10 mM)	344 ± 2

The results were somewhat different when EDTA was tested; EDTA cannot penetrate membranes easily [26] and does not inhibit RNA synthesis in yeast [27]. When EDTA was added to a final concentration of 5 mM at the time of maximum activity, a substantial increase in activity occurred over the next 4 h (Fig. 4). This increase did not occur when the other chelators were used, presumably because they inhibit RNA synthesis. (The kinetics of the increase after EDTA addition were similar to those observed during 'superderepression' of asparaginase II in exponential phase cells starved for nitrogen in the presence of an energy source [8]). About 5 h after addition of EDTA, cultures were in stationary phase, and loss of activity occurred. We have not yet investigated loss of asparaginase II activity in cells under these conditions.

As shown in Fig. 5, the activity retained by the cells 3 h after the addition of EDTA is linearly dependent upon the concentration of EDTA, up to 5 mM,

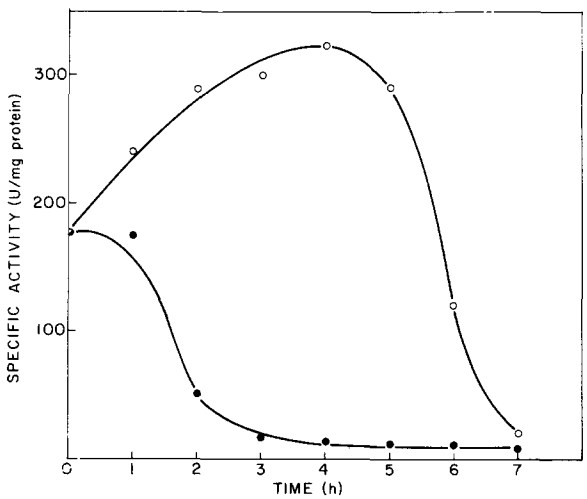

Fig. 4. Effect of 5 mM EDTA on the levels of asparaginase II in rapidly growing cells. Cells were grown in minimal medium to a density of $1.7 \cdot 10^7$ cells/ml, at which time EDTA was added. The control culture continued to grow in the original medium. Samples of the two cultures were removed at the indicated times and assayed immediately for asparaginase II. Control culture (●——●); EDTA (○——○).

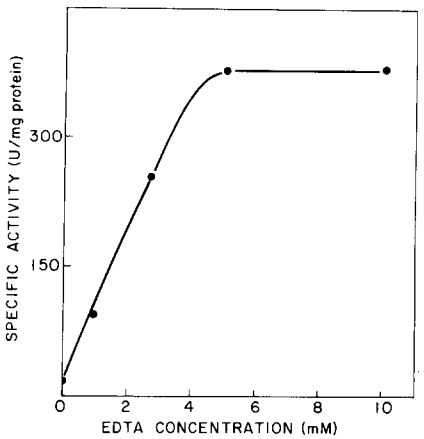

Fig. 5. Effect of increasing concentrations of EDTA on levels of asparaginase II. Cells were grown in minimal medium to a density of $1.7 \cdot 10^7$ cells/ml. The cultures then were supplemented with the indicated final concentrations of EDTA and incubated for 3 h. At this time, cells were harvested and assayed immediately for asparaginase II.

which is approximately the concentration of divalent cations in the minimal medium used in our laboratory [28]. The culture to which EDTA was added possessed an asparaginase II specific activity of about 180 U/mg protein. Thus, EDTA concentrations lower than 2.5 mM permit partial loss of activity, while concentrations greater than this allow an increase in activity.

Effect of EDTA and cycloheximide

We have shown that, during the hour before the loss of asparaginase II activity begins, protein synthesis is required for the process to occur [8]. It is pos-

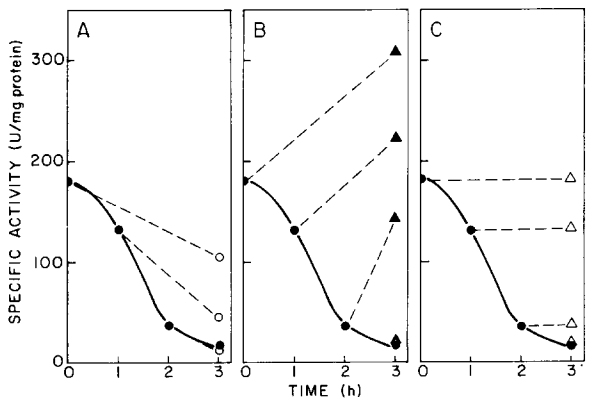

Fig. 6. Effect of concurrent addition of EDTA and cycloheximide on asparaginase II inactivation. Cells were grown in minimal medium to a density of $1.7 \cdot 10^7$ cells/ml. Either 100 μg/ml cycloheximide (panel A), 5 mM EDTA (panel B) or both (panel C) were added to aliquots of the culture at this time and subsequently at 1, 2 and 3 h. The unsupplemented control was assayed at the time of each addition. All of the supplemented subcultures were assayed 3 h after the first addition. Dashed lines indicate times on control curve at which inhibitor was added for each final assay point. They do not represent interpolations of enzyme activities. Control culture (●———●); cycloheximide (○———○); EDTA (▲———▲); EDTA and cycloheximide (△———△).

sible that a specific asparaginase II inhibitor is synthesized during this period and later in the growth cycle and that EDTA exerts its effect by disrupting an asparaginase II-inhibitor-metal ion complex. If so, addition of EDTA when protein synthesis is inhibited should increase the activity in cells in which a partial or total loss has occurred.

In the experiment shown in Fig. 6, either 5 mM EDTA or 100 μg/ml cycloheximide or both were added to aliquots of a culture at the time of maximum activity and at 1, 2 and 3 h after this. The control, allowed to grow as usual, was assayed at the time of each addition. All of the subcultures were assayed 3 h after the first addition. Activity relative to the control decreased during subsequent incubation when only cycloheximide was added and increased when only EDTA was added. No significant loss or gain relative to the control occurred when both inhibitors were added. Thus, EDTA does not act by dissociating an enzyme-inhibitor-metal ion complex. The results indicate that the increase observed after addition of EDTA is due to de novo protein synthesis. This conclusion is supported by the results obtained from the experiments in which the RNA synthesis inhibitors were added (Table III). The results also suggest that de novo synthesis of asparaginase II can occur long after the decline in activity has begun.

Reversal of EDTA effect by divalent cations

Because EDTA is a metal chelating agent, it should be possible to reverse its action by adding back the metal ion required for inactivation to occur. This possibility was tested in two different ways.

In the first method, cultures at the point of maximum activity were made 5 mM in EDTA (Table IV). After an incubation period of 10 min, various concentrations of cations were added to aliquots of the EDTA-treated culture. The control culture was allowed to grow with no additions except for a small amount of water to correct for volume changes. Activities were measured 3 h later. In Table IV, the activity measured in the EDTA-treated culture to which no additions had been made is considered to be 100%. Addition of Ca^{2+} or

TABLE IV

REVERSAL OF EDTA EFFECT IN GROWING CULTURES

EDTA was added to cultures at a density of $1.7 \cdot 10^7$ cells/ml. After an incubation period of 10 min, concentrated stock solutions of metal salts were added to aliquots of the EDTA-treated culture. The cells were harvested and assayed 3 h later. N.T., not tested.

Addition	% remaining activity		
none	7		
5 mM EDTA	100		
5 mM EDTA, followed by:	1 mM *	2 mM *	5 mM *
Zn^{2+}	72	14	6
Cu^{2+}	84	19	8
Co^{2+}	62	N.T.	6
Mn^{2+}	76	18	15
Ca^{2+}	102	N.T.	91
Mg^{2+}	131	N.T.	104

* Final cation concentration.

TABLE V

REVERSAL OF EDTA EFFECT BY DIVALENT CATIONS

EDTA was added to cultures at a density of $1.7 \cdot 10^7$ cells/ml. After an incubation time of 10 min, cells were harvested, washed, and resuspended in buffer containing different metal salts. The control culture was not supplemented. The cells were harvested and assayed after 3 h incubation in buffer or minimal medium. Control activities (U/mg protein): cells in minimal medium at $1.7 \cdot 10^7$ cells/ml: Expt. 1, 160 ± 4, Expt. 2, 128 ± 6; cells in minimal medium 10 min after addition of EDTA: Expt. 1, 166 ± 4, Expt. 2, 140 ± 4; cells in minimal medium grown to $3.8 \cdot 10^7$ cells/ml: Expt. 1, 18 ± 0.3, Expt. 2, 19 ± 0. In Expt. 1 the buffer was 20 mM Pipes (pH 7) and in Expt. 2 it was 20 mM Hepes (pH 7). Values shown represent the mean ± the S.E. of the mean of triplicates.

Incubation medium	Asparaginase II specific activity (units/mg protein)	
	Expt. 1	Expt. 2
Buffer	175 ± 3	144 ± 2
Buffer + 5 mM EDTA	157 ± 2	N.T.
Buffer + 5 mM Ca^{2+}	167 ± 4	N.T.
Buffer + 5 mM Mg^{2+}	169 ± 4	N.T.
Buffer + 5 mM Mn^{2+}	169 ± 1	N.T.
Buffer + 1 mM Co^{2+}	N.T.	145 ± 1
Buffer + 1 mM Zn^{2+}	N.T.	61 ± 2
Buffer + 5 mM Zn^{2+}	30 ± 1	N.T.

Mg^{2+} had essentially no effect on the inhibitory action of EDTA, whereas addition of Cu^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+} allowed normal loss to occur. These experiments show that addition of divalent metal ions can reverse the effects of EDTA in growing cells.

Because these experiments were done in a defined organic medium containing all the elements necessary for growth of *S. cerevisiae*, we could not tell from them which cation(s) is essential for loss of activity. Therefore, we performed a second series of experiments as follows. Cells were treated with EDTA for 10 min, as described above, but were then washed with either 20 mM Pipes (pH 7) or 20 mM Hepes (pH 7). (The same results were obtained with either buffer.) After they were washed, the cells were resuspended in buffer containing different metal salts. After 3 h incubation the cells were assayed for asparaginase II. (It was not possible to measure the specific activity of cells incubated in buffer plus Cu^{2+}, because the presence of this cation in the buffer caused the cells to clump irreversibly.) The results presented in Table V show that inactivation occurred only in the buffer containing Zn^{2+}. We have found in other experiments that Zn^{2+} is not an inhibitor of asparaginase II (data not shown). The results of these experiments suggest that inactivation of asparaginase II is a Zn^{2+}-dependent process.

Effect of mutation in carboxypeptidase S on inactivation of asparaginase II

Carboxypeptidase S is a Zn^{2+}-dependent peptidase of *S. cerevisiae* that has recently been discovered by Wolf and coworkers [13,14]. To test the possible involvement of this enzyme in the loss of asparaginase II, we crossed a mutant lacking carboxypeptidase S with our wild type strain S288C. We found that asparaginase II loss occurs normally in segregants that lack carboxypeptidase S (data not shown).

Discussion

The loss of asparaginase II just before the culture undergoes the transition to stationary phase appears to be the result of in situ degradation or inactivation. Although our failure to find activity in the medium or sequestered in the cell does not prove that one of these processes does not take place, the evidence presented in this paper makes these mechanisms most unlikely.

The most appealing suggestion to account for the loss is that it is the result of proteolytic degradation of the enzyme. This is supported by several lines of evidence. First, the fact that the specific protease inhibitor TPCK can inhibit loss of activity is strong evidence for this mechanism. The observation that protein synthesis is required shortly before the loss of activity begins suggests that one or more newly synthesized proteins is involved in the loss. It is unlikely that this protein(s) is a kinase that could inactivate asparaginase II by phosphorylation, because Mg^{2+} or Mn^{2+} alone cannot reverse the effect of EDTA [29]. Similarly, it is unlikely that α-mannosidase is involved, because this enzyme is not metal dependent in yeast [30]. Finally, the observation that concurrent addition of cycloheximide and EDTA causes activity to remain at control levels suggests that the newly synthesized protein is not a specific inhibitor that inactivates asparaginase II by forming a reversible enzyme-inhibitor-Zn^{2+} complex. An alternative explanation for this observation is that once the putative complex is formed, the enzyme is irreversibly inhibited.

The internal proteases of yeast have been studied intensively [9,20]. With our inhibitor studies, we have eliminated the involvement of all the well characterized internal proteases in the loss of asparaginase II. Our results with asparaginase II are similar to those of Ciejek and Thorner [12], who found that none of these proteases is involved in the extracellular degradation of α-factor, although the enzyme(s) involved in this process do not appear to be involved in the inactivation of asparaginase II (Table II).

Several peptidases that are Zn^{2+} dependent and *p*-hydroxymercuribenzoate sensitive have been described in yeast. One of these is peptidase α [31], a carboxypeptidase that is probably the same as carboxypeptidase S of Wolf and coworkers [13,14]. We have found that cells lacking carboxypeptidase S [14] lose asparaginase II activity in the same manner as wild type cells possessing carboxypeptidase S activity. Thus, this carboxypeptidase does not appear to be involved. One or more Zn^{2+}-dependent, *p*-hydroxymercuribenzoate-sensitive aminopeptidases exists in yeast [32]. The possible role that these enzymes might play in the inactivation of asparaginase II will await the isolation of mutants lacking these aminopeptidases.

The observation that only Zn^{2+} can reverse the EDTA effect in buffer, while Mn^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} are effective in culture medium can most likely be explained by examining the stability constants of EDTA-metal ion complexes [33]. The stability constant for Ca^{2+}- or Mg^{2+}-EDTA complexes is very low compared to those for the other ions. Thus when these cations are added to the EDTA-treated growth medium, they are not able to displace enough of the Zn^{2+} complexes with EDTA for the reversal to occur. The stability constants for the other complexes are much higher, so that it is likely that when they are added, enough Zn^{2+} is displaced to allow the reversal to occur.

Finally, the results we obtained when we tested *o*-phenanthroline, 8-hydroxyquinoline, and EDTA should serve as a caveat to future investigators who plan to use *o*-phenanthroline or 8-hydroxyquinoline as RNA synthesis inhibitors. In view of the many divalent cation-dependent processes that occur in the cell, it is probable that using chelators such as these or lomofungin [27] to inhibit RNA synthesis will have multiple effects.

In conclusion, our results suggest that the loss of asparaginase II activity during the transition between exponential and stationary phases is the result of proteolytic degradation. Although proteolysis of yeast internal enzymes in response to changing environmental or nutritional conditions has been studied extensively [9,10], we believe that this is the first example of such a mechanism for an external enzyme of *S. cerevisiae*.

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