Asparaginase II of Saccharomyces cerevisiae: Dynamics of Accumulation and Loss in Rapidly Growing Cells

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Asparaginase II activity in Saccharomyces cerevisiae can be derepressed in stationary phase cells by nitrogen starvation in the presence of an energy source. We have found that high activity of this enzyme is present in early-exponential phase cells even in the presence of abundant nitrogen. In growing cells that contain high asparaginase II activity, further derepression by nitrogen results in the rapid appearance of additional activity. Rapid loss of activity occurs as cultures begin to emerge from exponential growth. Synthesis of protein is required just before loss of activity occurs. Supplementing cultures with L-asparagine or L-glutamine strongly affects the kinetics of loss of activity. Mutation in ASP2 or ASP3, which results in inability to derepress this enzyme in stationary phase cells, also prohibits the development of the enzyme in exponentially growing cells.

INTRODUCTION

Regulation of the synthesis, utilization and degradation of L-asparagine is important to the growth and development of both plants and animals. In plants, L-asparagine metabolism is closely connected with the fixation and assimilation of atmospheric nitrogen (Atkins et al., 1975; Scott et al., 1976). In several types of animal cells, L-asparaginases, which deamidate L-asparagine, have been shown to exhibit marked antimitotic activity (for review, see Wriston & Yellin, 1973). Little is known about the regulation of asparagine metabolism in these systems.

In Saccharomyces cerevisiae the regulation of asparagine metabolism is complex. L-Asparagine synthesis is affected in an unknown way by the products of two unlinked cistrons (ASN1 and ASN2), mutation in each of which is required for L-asparagine auxotrophy (Jones, 1978). L-Asparaginase degradation is accomplished by two distinct forms of asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) in this species. L-Asparaginase I is synthesized constitutively and is active only on intracellular L-asparagine (Jones & Mortimer, 1973). Asparaginase II, which has been extensively characterized by its discoverers (Dunlop & Roon, 1975; Dunlop et al., 1976, 1978), is a glycoprotein that can deamidate both L- and D-asparagine when they are external to the cell. Asparaginase II can be strongly derepressed by incubating cells from dense cultures in medium containing an energy source but no nitrogen source (Dunlop & Roon, 1975; Dunlop et al., 1978). This enzyme is one of very few derepressible, externally active enzymes known in yeast.

Biochemical and genetic evidence shows that L-asparaginase I and asparaginase II are not closely related structurally. L-Asparaginase I is coded for by a single cistron (ASPI),
whereas at least two unlinked cistrons (ASP2 and ASP3) are involved in the expression of asparaginase II activity (Jones, 1973, 1977a; Jones & Mortimer, 1970). However, the two enzymes do interact in some way to permit efficient use of asparagine as a nitrogen source for growth (Jones, 1977b). When L-asparaginase I is present in cells, their growth rate in medium containing L-asparagine as the only nitrogen source is independent of the ability to express asparaginase II activity. In contrast, strains possessing only asparaginase II grow more slowly in this medium than when both enzyme activities are present. In medium containing D-asparagine as a sole nitrogen source, cells lacking L-asparaginase I activity but possessing asparaginase II grow more rapidly than cells containing both enzymes, even though D-asparagine is an extremely poor substrate for L-asparaginase I (Dunlop et al., 1978; Jones, 1977b). These observations indicate that interactions between the asparaginases might be an important aspect of growth regulation, but not enough is known about the physiological functions of these enzymes to allow the construction of models describing their behaviour.

Mechanisms governing the synthesis of asparaginase II in yeast have been studied only in cells in stationary phase cultures and, therefore, in cells with low physiological activity. Important questions are: what are the physiological roles of this enzyme during active growth of cells? Is the presence of asparaginase II simply a response to nitrogen starvation? Is its major role to salvage nitrogen in the form of asparagine? Is the presence of the enzyme affected by nutritional conditions other than the presence or absence of nitrogen? In this paper, we describe the behaviour of asparaginase II in cells growing rapidly in medium containing abundant nitrogen. Our results show that the physiological function of this enzyme is more subtle than simply providing a nitrogen salvage mechanism.

METHODS

Yeast strains. Saccharomyces cerevisiae was used. Strain S288C was obtained from the Yeast Genetics Stock Center (University of California, Berkeley, California), cloned and used as the wild-type strain in all experiments reported here. Strains carrying mutations in ASP1, ASP2 and ASP3 (see Table 1) were derived from mutants described by Jones & Mortimer (1970) and Jones (1977a) by repeatedly backcrossing mutant strains bearing different combinations of the altered genes. Genotypes of these strains are given in Table 1. Gene symbols have been described (Jones, 1977a; Jones & Mortimer, 1970).

Media. Minimal medium with vitamins (MV; Jones & Mortimer, 1973), sterilized by filtration, was used for cell propagation. In this medium, nitrogen is present as free ammonium ion (75 mm). Concentrated stocks of glucose, ammonium sulphate or amino acids were sterilized by filtration and added to culture media when required.

Growth of cells. Cells were grown at 29 ± 1°C on rotary shakers operated at 190 rev. min⁻¹. Culture turbidity at 520 nm was used to indicate cell titres. Turbidity was measured in 22 mm (o.d.) culture tubes in a Spectronic 20 (Bausch & Lomb) spectrophotometer. Cell titres were also determined by counting cells in a haemocytometer.

Cell preparation and enzyme activity determination. Unstarved cells were harvested by filtration, washed three times with 20 mm-potassium phosphate buffer, pH 7.0, resuspended in this buffer and assayed immediately for asparaginase II activity. To derepress cells by nitrogen starvation (Dunlop & Roon, 1975; Dunlop et al., 1976, 1978) they were suspended in 20 mm-potassium phosphate buffer, pH 7.0, containing 3% (w/v) glucose (derepression medium) and incubated on a rotary shaker at 29 ± 1°C. Derepressed cells were prepared for asparaginase II assay as described above.

Asparaginase II activity was determined by a modification of the whole cell assay described by Dunlop & Roon (1975). The densities of cell suspensions were adjusted so that the total activity measured was linear with time. All assays were performed in triplicate. D-Asparagine was added to the cell suspensions at a final concentration of 10 mm. After 30 min at 23°C, mixtures were placed in a 100°C water bath for 6 min to stop the reactions. The samples were then centrifuged to remove dead cells.

Ammonium ion concentrations in the supernatants were determined spectrophotometrically by coupling with glutamate dehydrogenase (Kaltwasser & Schlegel, 1966). Assay mixtures contained, in a final volume of 1-0 ml, 0-4 ml of an ammonium ion sample, 5-65 units glutamate dehydrogenase (Sigma), 200 nmol NADH (Sigma), 0-8 μmol 2-oxoglutarate (Sigma) and 0-6 ml 0-2 M-potassium phosphate buffer, pH 7-4.
Asparaginase II in growing yeast cells

Fig. 1. Time course of asparaginase II specific activity accumulation and loss in cells growing in MV medium. Stationary phase cells were seeded into fresh medium to an initial density of $9 \times 10^5$ cells ml\(^{-1}\). Growth conditions were as described in the text. Samples were removed at the indicated times and immediately assayed for asparaginase II activity. Error bars representing one standard error are shown when the standard error of the mean of triplicate samples exceeds the size of the symbol. Culture density, expressed as number of cells ml\(^{-1}\) (○); asparaginase II specific activity (●).

Fig. 2. 'Superderepression' of asparaginase II in growing cells. Cells were grown in MV medium to a density of $1.7 \times 10^7$ cells ml\(^{-1}\). A portion was then harvested, washed and resuspended in 20 mM-potassium phosphate buffer, pH 7.0, with or without 3% (w/v) glucose. The remainder was allowed to continue to grow in the original MV medium. Samples of the three cultures were removed at the times indicated and immediately assayed for asparaginase II specific activity. Error bars are shown when the standard error of the mean of triplicate samples exceeds the size of the symbol. Cells allowed to continue growth in MV medium (○); cells incubated in buffer without glucose (●); cells incubated in buffer containing glucose (△).

The reaction mixtures were incubated for 1 h at 23 °C, and the change in absorbance at 366 nm was measured in a Beckman Acta CII spectrophotometer. The assay was linear for ammonium ion concentrations between 0 and 200 μM.

Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard. To extract proteins, cells were centrifuged and resuspended in 1 M-NaOH. This suspension was frozen, thawed and placed in a 100 °C water bath for 10 min.

One unit of asparaginase II activity (U) produces 1 nmol ammonium ion min\(^{-1}\).

RESULTS

Asparaginase II activity in rapidly growing cells

To determine whether asparaginase II activity is expressed under physiological growth conditions, cells from an overnight stationary phase culture (about $8 \times 10^7$ cells ml\(^{-1}\)) were diluted into fresh MV medium to a density of about $9 \times 10^6$ cells ml\(^{-1}\) and allowed to grow. Whole-cell specific activity of asparaginase II was assayed periodically during the growth of the culture (Fig. 1). Immediately after dilution, activity averaged about 11 U (mg protein\(^{-1}\)), which is similar to the activities observed in repressed stationary phase cultures by Dunlop & Roon (1975). Following an initial lag of about 1.5 h, specific activity increased substantially as cells entered and progressed through exponential growth, reaching a maximum 9 to 10 h after dilution of the dense culture. At this time, the cell concentration was about $1.3 \times 10^7$ to $1.8 \times 10^7$ cells ml\(^{-1}\). Over the next 2 h, during which the cell density increased...
only about twofold, asparaginase II specific activity declined dramatically to the level initially observed in the stationary phase culture. At the time of loss of asparaginase II activity, the growth rate of the culture began to decline, although the stationary phase was not reached for at least another 7 h.

In several such experiments, the kinetics of appearance and loss of asparaginase II activity were nearly identical, although the maximum specific activity attained varied from experiment to experiment by about 25%. An important feature of these experiments is that asparaginase II activity is expressed per mg protein. Thus, these changes represent genuine changes in specific activity of the enzyme as measured in whole cells.

'Superderepression' of asparaginase II activity in actively growing cells

Asparaginase II activity reaches a maximum of about 200 to 220 U (mg protein)$^{-1}$ during exponential growth. About the same specific activity is obtained when stationary phase cells are derepressed by nitrogen starvation (Dunlop & Roon, 1975; personal observations in our laboratory). Is this therefore the maximum specific activity that can be elicited under any conditions, or do cells possess reserve capacity to synthesize or mobilize more of the enzyme during physiological stress? To answer this question, we determined whether cells in which asparaginase II activity was already present could be further derepressed by nitrogen starvation. Cells from an exponentially growing culture in which maximum asparaginase II activity had been attained were washed and resuspended in derepression medium. As controls, cells from the same culture were suspended in buffer without glucose, and cells in a third portion of the culture were allowed to continue growing undisturbed. Asparaginase II specific activities in these three cultures were determined periodically (Fig. 2). Under these conditions, asparaginase II was substantially derepressed in buffer with the energy source, reaching a maximum of about 350 U (mg protein)$^{-1}$ after about 4 h derepression. Activity in the control culture containing only buffer declined by about 40% over 2 h and then remained constant at about 100 U (mg protein)$^{-1}$. As expected, cells allowed to continue growth in MV medium had lost almost all activity 2 h after the beginning of the experiment. Thus, not only was asparaginase II further derepressed by nitrogen starvation of actively growing cells, but withholding nitrogen and energy sources from those cells prevented much of the loss of activity that normally would have occurred during their subsequent growth.

Effects of mutation in ASPI, ASP2 and ASP3 on the expression of asparaginase II

in rapidly growing cells

It is possible that the asparaginase II activity detected in exponentially growing cultures is not the same as that detected in derepressed stationary phase cells. To resolve this question, the effects of mutation in ASPI, ASP2 and ASP3 on specific activities in rapidly growing cells were examined using highly inbred strains carrying various combinations of mutations in these genes. As shown in Table 1, a mutation in ASP2 or ASP3 essentially abolished the appearance of asparaginase II activity in cells from all the cultures tested. Mutation in ASPI, the structural gene for L-asparaginase I, had no effect on the expression of asparaginase II. Thus, asparaginase II activity measured in rapidly growing cells was the same as that observed in derepressed stationary phase cells; both activities were abolished by mutations in the same genes.

Effects of nitrogen and energy sources on the loss of asparaginase II activity

from rapidly growing cells

The dramatic loss of asparaginase II activity from whole cells just as they begin to emerge from exponential growth suggests that changes in nutritional state might be responsible for triggering the loss. We attempted to modify the loss of activity by adding metabolites to
Table 1. Asparaginase II activity of ASP1, ASP2 and ASP3 mutants

Cells were grown in MV medium to a density of 1.7 x 10⁷ cells ml⁻¹ (exponential phase) or 3.8 x 10⁷ cells ml⁻¹ (transition phase) and immediately assayed. The asparaginase II specific activities represent the mean ± standard error of the mean of triplicate samples.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Exponential phase cultures</th>
<th>Transition phase cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>S288C</td>
<td>wild-type</td>
<td>228±3</td>
<td>10±1</td>
</tr>
<tr>
<td>XE243-1B</td>
<td>asp1-12</td>
<td>207±4</td>
<td>10±1</td>
</tr>
<tr>
<td>XE221-2A</td>
<td>asp2</td>
<td>15±2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>XE223-1C</td>
<td>asp3</td>
<td>7±1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>XE225-3A</td>
<td>asp1-12 asp2</td>
<td>18±1</td>
<td>5±1</td>
</tr>
<tr>
<td>XE226-2D</td>
<td>asp1-12 asp3</td>
<td>10±1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>XE216-5B</td>
<td>asp2 asp3</td>
<td>10±1</td>
<td>&lt;5</td>
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<tr>
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<td>asp1-12 asp2 asp3</td>
<td>6±1</td>
<td>6±1</td>
</tr>
</tbody>
</table>

Table 2. Effect of supplementation of the culture medium on the loss of asparaginase II activity

Cultures were supplemented at a density of 1.7 x 10⁷ cells ml⁻¹ and allowed to grow to a density of 3.8 x 10⁷ cells ml⁻¹. At this time cells were harvested and immediately assayed. Cultures were made 5 mM in L-aspartate by adding crystals of L-aspartate. Concentrated stock solutions were added to the other cultures. The asparaginase II specific activities shown represent the mean ± standard error of the mean of triplicate samples.

<table>
<thead>
<tr>
<th>Supplement (final concn)</th>
<th>Specific activity [U (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11±1</td>
</tr>
<tr>
<td>D-Glucose (55.5 mM)</td>
<td>11±1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (37.5 mM)</td>
<td>11±1</td>
</tr>
<tr>
<td>L-Asparagine (5 mM)</td>
<td>81±4</td>
</tr>
<tr>
<td>L-Glutamine (5 mM)</td>
<td>65±3</td>
</tr>
<tr>
<td>D-Asparagine (3.3 mM)</td>
<td>10±1</td>
</tr>
<tr>
<td>L-Aspartate (5 mM)</td>
<td>26±1</td>
</tr>
<tr>
<td>L-Glutamate (5 mM)</td>
<td>7±1</td>
</tr>
<tr>
<td>L-Arginine (5 mM)</td>
<td>9±1</td>
</tr>
<tr>
<td>L-Proline (5 mM)</td>
<td>8±1</td>
</tr>
</tbody>
</table>

cultures immediately before the decline should have occurred. Metabolites chosen were glucose (the usual carbon source in MV medium), ammonium sulphate (the usual nitrogen source in this medium), and several amino acids that can be used as sole nitrogen sources for growth. As shown in Table 2, supplementing cultures with glucose, ammonium ion, L-proline, L-arginine, L-glutamate or D-asparagine had no effect on the loss of activity during subsequent growth, and L-aspartate had only a small effect. In marked contrast, addition of L-asparagine or L-glutamine to cultures had profound effects on the disappearance of activity from whole cells, and the degree of effectiveness was strongly dependent on the concentration of amino acid added (Fig. 3). Maximum effect on the loss of asparaginase II specific activity did not occur until the concentration of L-asparagine reached about 8 mM. L-Glutamine was somewhat less effective; about 2 mM more of this amino acid was required to affect loss to the same extent as L-asparagine. Above 10 mM, L-asparagine and L-glutamine effects were equal and maximal.

To determine how these amino acids affected the loss of asparaginase II activity, the following experiment was done. An exponentially growing culture was divided into three subcultures at the time of maximum asparaginase II activity. Either L-asparagine or
Fig. 3. Effect of increasing concentrations of L-asparagine and L-glutamine on the loss of activity of asparaginase II from rapidly growing cells. Cells were grown in MV medium to a density of $1.7 \times 10^7$ cells ml$^{-1}$. Cultures were then supplemented with the indicated final concentrations of L-asparagine or L-glutamine and allowed to grow to a density of $3.8 \times 10^7$ cells ml$^{-1}$. At this time, cells were harvested and assayed immediately for asparaginase II activity. To achieve concentrations of L-asparagine above 10 mM or L-glutamine above 15 mM, crystals of the amino acids were slowly added to vigorously stirred cultures. For lower concentrations, concentrated stock solutions were added to the cultures. L-Asparagine (○); L-glutamine (●).

Fig. 4. Effect of 10 mM L-asparagine or L-glutamine on the kinetics of loss of asparaginase II from rapidly growing cells. Cells were grown in MV medium to a density of $1.7 \times 10^7$ cells ml$^{-1}$. Cultures were then supplemented with either L-asparagine or L-glutamine at 10 mM. The control culture continued to grow in the original medium. Samples of the three cultures were removed at the times indicated and assayed immediately for asparaginase II specific activity. Control culture (●); culture supplemented with L-asparagine (○); culture supplemented with L-glutamine (▲).

L-glutamine at a final concentration of 10 mM was added to two of the subcultures. The third subculture (control) was allowed to continue to grow in unsupplemented minimal medium. Samples were withdrawn at intervals and assayed for whole-cell asparaginase II activity (Fig. 4). As expected, specific activity in the unsupplemented control culture dropped rapidly within a period of about 2 h. In contrast, the kinetics of the loss of activity in the supplemented cultures were changed dramatically. In the cultures to which 10 mM L-asparagine or L-glutamine was added, activity was lost much more slowly than in the control culture. At 3 h (the time used in the experiment described in Fig. 3), control cultures had lost nearly all activity, but the supplemented cultures retained nearly half the original activity. About 6 to 7 h were required for complete loss in the supplemented cultures. The time of initiation of loss and final specific activities were nearly the same in all cultures. Thus the amide amino acids affected only the rate of loss of activity.

**Relationship of the loss of asparaginase II activity to protein synthesis**

When growing cells containing peak activity were suspended in buffer without an energy source, not all the activity was lost during incubation (see above). This observation suggests that loss of the enzyme is an active process, perhaps requiring protein synthesis. To test this hypothesis, cells were grown to an absorbance of 0.25 (about $1.1 \times 10^7$ cells ml$^{-1}$) at which time asparaginase II specific activity was about 185 U (mg protein)$^{-1}$ (Fig. 5, inset). Cycloheximide (100 μg ml$^{-1}$) (CY) was added to samples of the culture at this time (time 0 of the experiment) and at increasing intervals of additional growth. The asparaginase II specific activity of these cultures was measured 4.5 h after the start of the experiment (Fig. 5).
Asparaginase II activity in the untreated cells was determined periodically for 4.5 h (Fig. 5, inset). As expected, activity in the control culture increased to a maximum of about 210 U (mg protein)^{-1} at 1.5 h and then, over 2 h, decreased to about 8 U (mg protein)^{-1} and remained constant until 4.5 h, when the experiment was terminated. Over this time, the protein concentration in the control culture increased exponentially from 0.04 to 0.15 mg ml^{-1} (results not shown).

In cultures to which CY was added, protein concentrations at 4.5 h were not significantly different from concentrations in those cultures at the time when CY was added (results not shown). Thus, CY rapidly and effectively inhibited net protein synthesis, and asparaginase II activities at 4.5 h were markedly dependent on the time of CY addition.

The important aspects of this experiment were: (i) when protein synthesis was inhibited at \( t = 0 \) or 0.75 h before the peak occurred, there was no loss of activity; (ii) when CY was added at the time of peak activity \( (t = 1.5 \text{ h}) \), much of the expected loss occurred, and only about 95 U (mg protein)^{-1} remained at the end of the experiment; and (iii) once the decline had begun, nearly all the loss occurred despite the addition of CY to the cultures.

**DISCUSSION**

We have shown that ability to respond to nitrogen starvation accounts for only part of the physiological function of asparaginase II in yeast. High activity of the enzyme is present in cells in the early phase of exponential growth even in the presence of abundant nitrogen in the form of free ammonium ion. At a critical point in the growth of cultures, asparaginase II specific activity drops precipitously over a time interval of about one cell generation.

The 'superderepression' of asparaginase II observed in our experiments is similar to the derepression of this enzyme by nitrogen starvation of cells in stationary phase cultures as originally described by Dunlop & Roon (1975). Our results indicate that the regulatory mechanisms involved in the accumulation of the enzyme during exponential growth are different from that (those) involved in derepression by nitrogen starvation. That such might be the case is also indicated by the different kinetics of the appearance of the enzyme under the two conditions.
The sharp decrease in asparaginase II activity as culture density increases beyond about $2 \times 10^7$ cells ml$^{-1}$ is not the result of inactivation brought about by depletion of glucose or ammonium ion from the medium. The strong, specific responses shown when L-asparagine or L-glutamine is added to the medium suggest that loss of activity results directly or indirectly from changes in the internal nitrogen balance of cells as effected through the metabolism or compartmentalization of the amide amino acids. The experiments with cycloheximide show that protein synthesis is associated with the subsequent loss of activity of the enzyme. Once loss of activity has begun, however, protein synthesis is not required for loss to go to completion. Although the molecular mechanism governing the loss of activity remains unknown, these findings show that the cell can respond in a specific, active manner to environmental signals that trigger the loss, and that these processes are highly sensitive to the external, and probably, internal concentrations of amide amino acids.

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REFERENCES


