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Utilization of D-Asparagine by *Saccharomyces cerevisiae*

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Yeast strains Σ1278b and Harden and Young, which synthesize only an internal constitutive form of L-asparaginase, do not grow on D-asparagine as a sole source of nitrogen, and whole cell suspensions of these strains do not hydrolyze D-asparagine. Strains X2180-A2 and D273-10B, which possess an externally active form of asparaginase, are able to grow slowly on D-asparagine, and nitrogen-starved suspensions of these strains exhibit high activity toward the D-isomer. Nitrogen starvation of strain X2180-A2 results in coordinate increase of D- and L-asparaginase activity; the specific activity observed for the D-isomer is ~20% greater than that observed for the L-isomer. It was observed, in studies with cell extracts, that hydrolysis of D-asparagine occurred only with extracts from nitrogen-starved cells of strains that synthesize the external form of asparaginase. Furthermore, the activity of the extracts toward the D-isomer was always higher than that observed with the L-isomer. A 400-fold purified preparation of external asparaginase from *Saccharomyces cerevisiae* X2180-A2 hydrolyzed D-asparagine with an apparent K_m of 0.23 mM and a V_{max} of 38.7 μmol/min per mg of protein. D-Asparagine was a competitive inhibitor of L-asparagine hydrolysis and the K_i determined for this inhibition was approximately equal to its K_m. These data suggest that D-asparagine is a good substrate for the external yeast asparaginase but is a poor substrate for the internal enzyme.

The occurrence of an externally active asparaginase in certain strains of *Saccharomyces cerevisiae* was recently reported by our laboratory (6). The data we presented suggested that there are two classes of *Saccharomyces*. The first class possesses two forms of asparaginase: an externally active enzyme that is derepressed during nitrogen starvation and an internally active asparaginase that occurs constitutively. The second class of yeast possesses only an internal, constitutive form of asparaginase.

During the course of our investigation of the yeast strains that synthesize the external enzyme, it was found that mutants having a greatly reduced ability to transport L-asparagine could be obtained by selecting cells capable of rapid growth on low levels of D-asparagine as the sole source of nitrogen (6). These mutant strains were given the temporary designation of D-asparagine resistant (D-Asn^R). Since it had been previously reported that *Saccharomyces cerevisiae* is incapable of utilizing D-amino acids as the sole nitrogen source (11, 13), we decided to investigate this phenomenon more extensively. The present communication presents data that establish the metabolic basis for the growth of *Saccharomyces* on D-asparagine and demonstrate a correlation between the growth response of various strains and the occurrence of the external form of asparaginase.

MATERIALS AND METHODS

Organism. The origin and characteristics of the wild-type yeast strains used in this investigation have been given previously (6). Mutant strains lacking the general amino acid permease (*gap-1*) and the specific ammonia transport system (*amt-1*, ammonia transport deficient; formerly designated Mea^R) were derived from strain X2180-A2 by methodology that has been reported elsewhere (6, 12).

Growth conditions and methods of cell preparation. The standard minimal medium contained (per liter): 20 g of D-glucose, 2 g of yeast nitrogen base (Difco) (without amino acids and ammonia sulfate), and a nitrogen source as given in the text. The conditions for the growth of *S. cerevisiae* on minimal ammonia medium and for the preparation of unstarved and nitrogen-starved cell suspensions have been reported previously (6).

Measurement of asparaginase activity. The hydrolysis of L-asparagine by cell suspensions and cell extracts was measured using a spectrophotometric assay for ammonia through coupling with L-glutamate dehydrogenase (EC 1.4.1.3) or a spectrophotometric assay for L-aspartate through coupling with L-glutamic oxalacetic transaminase (EC 2.6.1.1) and L-malic dehydrogenase (EC 1.1.1.37) as previously reported (6). The hydrolysis of D-asparagine was

monitored using the spectrophotometric ammonia assay. Activities are reported as nanomoles of substrate hydrolyzed per minute per milligram of cells (dry weight), nanomoles per minute per milligram of protein (crude extracts), or micromoles per minute per milligram of protein (purified enzyme).

Chemicals. The D-asparagine utilized in these experiments was obtained from Sigma or Calbiochem and was identified as being chromatographically homogeneous by the manufacturers. Three additional criteria were used to verify the purity of the D-isomer. (i) Measurements of optical rotation indicated that the compound was 97% ± 3% D-isomer (literature value, $[\alpha]_D^{20} = -33.2$ in 3 N HCl; observed value = −32.3). (ii) The compound was tested as a substrate for L-asparaginase II from *Escherichia coli* (purified enzyme provided by J. B. Howard) and was hydrolyzed at 6% the maximal rate found with the L-isomer. This corresponds to values given in the literature (3). (iii) Under conditions where D-asparagine was highly active in the coupled assay for ammonia, no release of L-aspartate could be detected in the coupled assay for L-aspartate using L-glutamic oxalacetic transaminase and L-malic dehydrogenase.

RESULTS

Growth of S. cerevisiae strains on L- and D-asparagine. The strain-specific growth response to L- and D-asparagine is shown in Table 1. All of the yeast strains tested grew with a doubling time of approximately 2.5 h on the L-isomer. Strains Σ1278b and Harden and Young, which synthesize only the internal constitutive form of asparaginase (6), did not grow in medium containing D-asparagine as the sole source of nitrogen. These results are in agreement with the findings of Rytka (13) that D-amino acids are growth inhibitory and do not serve as metabolizable nitrogen sources for *Saccharomyces*. However, strains D273-10B and X2180-A2, which synthesize an external form of asparaginase in response to nitrogen starvation as well as a constitutive internal form (6), grew slowly with D-asparagine as the sole nitrogen source, exhibiting doubling times of 3.9 and 5.2 h, respectively. In an effort to determine the mode by which these strains utilized the D-isomer, the growth response to D-asparagine was examined in three mutant strains (derived from X2180-A2), which were deficient in their ability to assimilate D-amino acids. Strain NR 202 (formerly designated D-Asn^{R2}) was selected on the basis of its ability to grow rapidly on D-asparagine as described previously (6). Strain AR 101 was selected for the ability to grow on minimal proline medium in the presence of D-alanine according to the methods of Rytka (13). Strain LR 402 was selected for the ability to grow on minimal proline medium in the presence of 10 mM L-lysine. All three strains lacked detectable activity for the general amino acid permease *(gap-1)* and complementation tests revealed that the three mutations were allelic (data not shown).

TABLE 1. *Growth response of yeast strains to L- and D-asparagine*

Strain (*S. cerevisiae*)	Generation time at 21 C (h)	
	L-Asparagine[a]	D-Asparagine[b]
Σ1278b (wt)[c]	2.4	ND[d]
Harden + Young (wt)	2.1	ND
D273-10B (wt)	2.5	3.9
X2180-A2 (wt)	2.7	5.2
NR 202 (*gap-1*)[e]	2.7	3.1
AR 101 (*gap-1*)[f]	2.6	3.2
LR 402 (*gap-1*)[g]	2.6	2.6
MR (*gap-1, amt-1*)[h]	3.0	ND

[a] Determined in standard minimal medium containing 10 mM L-asparagine as sole source of nitrogen.

[b] Determined in standard minimal medium containing 10 mM D-asparagine as sole source of nitrogen.

[c] wt, Wild type.

[d] ND, No detectable growth after 48 h of incubation.

[e] Derived from X2180-A2; selection based on ability to grow in standard minimal medium containing 2 mM D-asparagine as sole source of nitrogen.

[f] Derived from X2180-A2; selection based on ability to grow in standard minimal medium containing 5 mM L-proline plus 10 mM D-alanine.

[g] Derived from X2180-A2; selection based on ability to grow in standard minimal medium containing 5 mM L-proline plus 10 mM L-lysine.

[h] Derived from AR 101 (*gap-1*); selection based on ability to grow in standard minimal medium containing 2 mM L-glutamate plus 200 mM methylamine hydrochloride.

The growth response to D-asparagine was enhanced to a similar extent in each of the three mutant strains with the 5.2-h generation time reduced to a value close to that observed with the L-isomer. These data suggested that growth on D-asparagine was enhanced in the mutants because they retained a normal ability to transport ammonia released from D-asparagine by the external asparaginase but due to mutation had a reduced ability to transport the growth-inhibitory D-amino acids (D-asparagine and its hydrolysis product, D-aspartic acid).

In support of this hypothesis, it was found that strain MR, derived from strain AR 101, which is defective in ammonia transport *(amt-1)* as well as in the general amino acid permease *(gap-1)*, grows poorly on D-asparagine. The growth of this double mutant strain on L-asparagine results from the transport of L-aspartate by the low-velocity specific transport system for dicarboxylic amino acids (9), since the activity of this system is not affected by

either the *gap-1* or *amt-1* mutations (R. Roon, unpublished data).

Hydrolysis of L- and D-asparagine by cell suspensions of S. cerevisiae. Cell suspensions of the wild-type yeast strains were examined for the ability to hydrolyze D- and L-asparagine. Strains Σ1278b and Harden and Young had no detectable external asparaginase activity for either isomer, whereas strains X2180-A2 and D273-10B had high levels of activity toward both isomers after nitrogen starvation but low levels of activity prior to starvation (Table 2).

The time dependency for the appearance of external asparaginase activity was examined in cell suspensions of strain X2180-A2 with both isomers as substrates (Table 3). Little activity was detectable for either isomer in unstarved cells or in cells starved in the presence of 10 mg of cycloheximide per liter (not shown). The specific activity toward both isomers increased in parallel with time of nitrogen starvation, with that observed for the D-isomer appearing ~10 to 20% higher than that observed with the L-isomer at all time points tested.

Hydrolysis of L- and D-asparagine by cell extracts of S. cerevisiae. Crude extracts of the various strains were also examined for the ability to hydrolyze both isomers (Table 4). In strains Σ1278b and Harden and Young the constitutive internal asparaginase was active toward the L- but not the D-isomer. Similarly, extracts prepared from unstarved cells of X2180-A2 and D273-10B exhibited activity toward the L- but not the D-isomer. In contrast, extracts prepared from nitrogen-starved cells of the latter yeast strains exhibited a high level of activity toward both isomers.

TABLE 2. *Hydrolysis of L- and D-asparagine by cell suspension of S. cerevisiae*

Strain (*S. cerevisiae*)	Asparaginase activity (nmol/min per mg of cells [dry wt])	
	L-Asparagine	D-Asparagine
X2180-A2		
Starved[a]	60.0	80.0
Unstarved[b]	<5.0	<5.0
D273-10B		
Starved	47.0	52.0
Unstarved	<2.0	<2.0
Σ1278b		
Starved	<2.0	<2.0
Unstarved	<2.0	<2.0
Harden + Young		
Starved	<2.0	<2.0
Unstarved	<2.0	<2.0

[a] Cells were incubated in nitrogen-free medium (20 mM potassium phosphate buffer [pH 7.0] and 3% glucose) for 3 h prior to assay.

[b] Ammonia-grown cells were suspended in medium and assayed immediately.

TABLE 3. *Appearance of external asparaginase activity of S. cerevisiae X2180-A2 as a function of time of nitrogen starvation*

Incubation time in nitrogen-free medium (min)	Asparaginase activity[a] (nmol/min per mg of cells [dry wt])	
	L-Asparagine	D-Asparagine
0	<2.0	<2.0
30	3.9	4.7
60	22.0	26.0
180	62.0	75.0

[a] Cells were incubated in nitrogen-free assay medium for the times indicated prior to assay.

TABLE 4. *Hydrolysis of L- and D-asparagine by cell extracts of S. cerevisiae*

Strain (*S. cerevisiae*)[a]	Asparaginase activity (nmol/min per mg of protein)	
	L-Asparagine	D-Asparagine
X2180-A2		
Starved	71.0	92
Unstarved	16.2	3.2
D273-10B		
Starved	37.1	36.8
Unstarved	13.5	<0.5
Σ1278b		
Starved	5.0	<0.5
Unstarved	6.6	<0.5
Harden + Young		
Starved	9.8	<0.5
Unstarved	10.6	<0.5

[a] Crude extracts of nitrogen-starved and unstarved cells were prepared as described in the text.

Hydrolysis of L- and D-asparagine by purified external asparaginase from cells of strain X2180-A2. The reaction kinetics of the hydrolysis of L- and D-asparagine were investigated using a highly (400-fold) purified preparation of the external asparaginase from *S. cerevisiae* X2180-A2 (purification procedure to be published elsewhere; P. C. Dunlop and R. J. Roon, manuscript in preparation). Substrate saturation data for both asparagine isomers with the external enzyme are shown in Fig. 1. Analysis of the data by the method of Lineweaver-Burk (5) yielded an apparent K_m of 0.23 mM and V_{max}

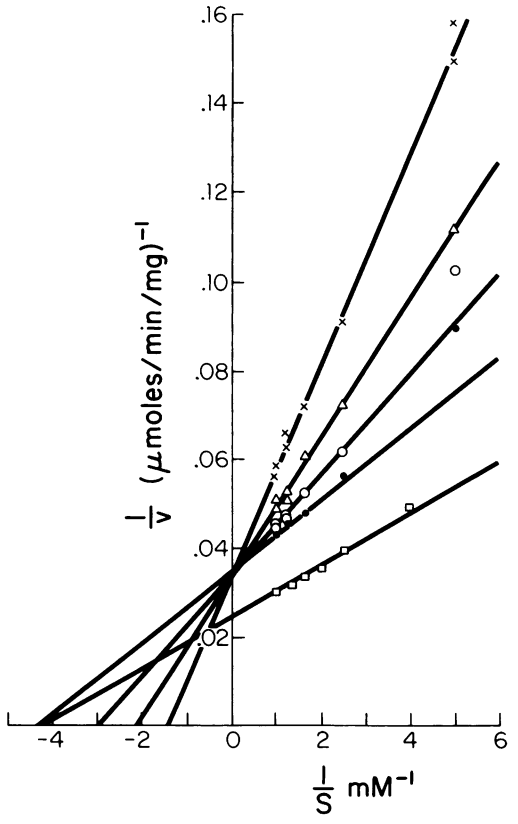

FIG. 1. *Lineweaver-Burk plot for the hydrolysis of* L- *and* D-*isomers of asparagine by purified external asparaginase from S. cerevisiae strain X2180-A2. The hydrolysis of* L-*asparagine at 21 C in the absence (●) and in the presence of* D-*asparagine (initial concentrations: 0.3 mM [○], 0.6 mM [△], and 1 mM [X]) was observed with the* L-*aspartate direct-couple spectrophotometric assay. The hydrolysis of* D-*asparagine (□) at 21 C was observed using the ammonia spectrophotometric assay.*

of 38.7 μmol/min per mg of protein for D-asparagine and an apparent K_m of 0.22 mM and V_{max} of 29 μmol/min per mg of protein for L-asparagine.

From the above results, two alternative hypotheses were formulated. Either the enzyme was nonspecific for the two isomers, with one active center involved for both substrates, or two enzymes existed which had co-purified, each with a specificity for one isomer. Two experimental approaches were undertaken to resolve this question.

The enzymic hydrolyses of substrate concentrations composed of varying proportions of L- and D-asparagine were observed using the glutamic dehydrogenase-coupled assay to monitor total ammonia produced. The reaction velocities observed are presented in Table 5. The data show "classical" mixed substrate velocities (5), which fall within a range that is between those observed for either substrate alone. Had the hydrolyses been due to two different enzymes, the observed rates should have been the sum of the separate rates. The ratio of the affinities of the enzyme for the two isomers, as defined by the equation $K_D/K_L = V_D - V_M/V_M - V_L$, where V_D and V_L equal maximum velocity attainable with D- and L- asparagine, respectively, and V_M equals the maximum velocity attainable with an equimolar mixture of D- and L-asparagine, was 1.07, indicating similar binding affinities.

The ability of D-asparagine to inhibit the hydrolysis of L-asparagine by the external enzyme was tested using the coupled spectrophotometric assay for L-aspartate. The data (Fig. 1) show that D-asparagine is a competitive inhibitor for L-asparagine hydrolysis. From the apparent K_m and K_m' values for the uninhibited and inhibited reactions, an apparent K_i for D-asparagine was calculated using the equation: $K_i = [i]/([K_m' = K_m] - 1)$. The K_i values thus calculated for D-asparagine were: 0.40 mM ($[i]$ = 1 mM), 0.5 mM ($[i]$ = 0.6 mM), and 0.79 mM ($[i]$ = 0.3 mM). The variation observed in K_i values is to be expected for a system in which the "inhibitor" simultaneously serves as a substrate, for at any time, t, the finite concentration, as well as

TABLE 5. *"Mixed substrate" velocities observed for external asparaginase[a] with asparagine isomers*

Substrate (concn, 1 mM)	Velocity (μmol/min per mg of protein)
100% L-asparagine	27.8[b]
80% L-asparagine 20% D-asparagine	30.0
60% L-asparagine 40% D-asparagine	31.6
50% L-asparagine 50% D-asparagine	32.3[c]
40% L-asparagine 60% D-asparagine	33.2
20% L-asparagine 80% D-asparagine	35.2
100% D-asparagine	37.1[d]

[a] Enzyme preparation was purified 400-fold. Glutamic dehydrogenase-coupled assay was used to monitor ammonia production.

[b,c,d] Velocities involved in the calculation of the ratio of affinities of the enzyme for the two isomers: K_D/K_L = (37.1 − 32.3)/(32.3 − 27.8) = 1.07.

proportionality, of inhibitor to substrate is changing. Therefore, to determine K_i at infinite inhibitor concentration, a plot of K_i observed versus the reciprocal of the inhibitor concentration was done. A straight line was observed, which intercepted the ordinate at $K_i = 0.24$ mM (not shown).

The data obtained from these two experimental approaches support the hypothesis that the two isomers are both substrates of a single form of asparaginase.

DISCUSSION

The following conclusions or inferences can be drawn from the data presented in this report.

(i) There appears to be a positive correlation between the ability of yeast strains to synthesize the external form of asparaginase and the growth response of these strains to D-asparagine as a sole nitrogen source; i.e., strains that synthesize the external enzyme are concomitantly able to utilize D-asparagine as a nitrogen source.

(ii) Internal L-asparaginase is inactive toward D-asparagine, but both the D- and L-isomers of asparagine appear to be excellent substrates for the external asparaginase.

(iii) General amino acid permeaseless mutants of *S. cerevisiae* X2180-A2 grow rapidly on D-asparagine, because the ammonia liberated from D-asparagine external to the cell can be transported into the cell at a normal rate but the uptake of D-asparagine and D-aspartate, which are growth inhibitors, is retarded.

(iv) Because of differences observed in their substrate specificities, it is unlikely that the internal and external forms of asparaginase exist in a simple precursor-product relationship; i.e., the two forms of asparaginase are probably not enzymes derived from the same basic protein molecule that differ only in their extent of postribosomal modification and/or ultimate site of action. Rather, it seems more likely that the two asparaginase enzymes will prove to be distinct protein molecules or multimeric enzymes that contain distinct polypeptide chains. An analogous situation appears to be true for the two alkaline phosphatases of yeast (16, 17) and may also prove to be true for the two forms of yeast invertase (7, 10).

Further areas of investigation have been motivated by the present study. The first of these is concerned with the substrate specificity of the external asparaginase. Preliminary evidence suggests that the substrate specificity of this enzyme is qualitatively similar to that of the *Erwinia* L-asparaginase reported by Howard and associates (8). However, there are dramatic quantitative differences in that various derivatives of asparagine with modifications of the α-amino group (e.g., *N*-acetyl L-asparagine) serve as excellent substrates for the yeast enzyme (P. C. Dunlop, unpublished data) but function only poorly as substrates for the *Erwinia* enzyme (8).

A second area of research prompted by this study involves the use of D-asparagine as a tool for selecting yeast mutants with an altered ability to secrete asparaginase. For example, since only the external enzyme is able to hydrolyze D-asparagine, it should be possible to selectively isolate mutants that lack external asparaginase activity based on their ability to grow on the L- but not the D-isomer. Genetic analysis of such mutants should give conclusive evidence for the nonidentity (or identity) of the two forms of yeast asparaginase.

Finally, the existence of two distinct enzyme forms suggests the need for a reevaluation of the clinical utility of yeast asparaginase. Although bacterial asparaginases have been used as chemotherapeutic agents against certain human tumors for a number of years (4), it was found in earlier studies that yeast asparaginase preparations were ineffective because of rapid clearance from the blood (12). However, it is unclear as to which form of the enzyme was used in these trials. In addition, since the external enzyme appears to be a mannan-glycoprotein (P. C. Dunlop, unpublished data), it may be possible to derive various forms of this molecule with modified carbohydrate content by presently available genetic (1, 15) and chemical (14) techniques. Alterations in the mannan content of the enzyme could result in dramatic differences in the rates of clearance.

It seems appropriate at this time to suggest alternate nomenclature for the two yeast asparaginases. The internal enzyme appears to function as a classical L-asparaginase; thus, it can appropriately be referred to as L-asparaginase I. Since the external enzyme shows a higher catalytic activity toward the D-isomer than the L-isomer, the designation L-asparaginase is inappropriate. Thus we would suggest that this enzyme be tentatively referred to as asparaginase II (external) without any stipulation of the isomer hydrolyzed. Further investigation may indicate that the term asparaginase itself is inappropriate in reference to this enzyme; however, our preliminary findings suggest that all of its optimal substrates are derived directly from the L-asparagine molecule. Unless further evidence suggests more appropriate nomenclature, the terms L-asparaginase I (internal) and asparaginase II (external) will be used to designate the two enzymes in subsequent reports.

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