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Regulation of the Nicotinamide Adenine Dinucleotide- and Nicotinamide Adenine Dinucleotide Phosphate-Dependent Glutamate Dehydrogenases of *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae contains two distinct L-glutamate dehydrogenases. These enzymes are affected in a reciprocal fashion by growth on ammonia or dicarboxylic amino acids as the nitrogen source. The specific activity of the nicotinamide adenine dinucleotide phosphate (NADP) (anabolic) enzyme is highest in ammonia-grown cells and is reduced in cells grown on glutamate or aspartate. Conversely, the specific activity of the nicotinamide adenine dinucleotide (NAD) (catabolic) glutamate dehydrogenase is highest in cells grown on glutamate or aspartate and is much lower in cells grown on ammonia. The specific activity of both enzymes is very low in nitrogen-starved yeast. Addition of the ammonia analogue methylamine to the growth medium reduces the specific activity of the NAD-dependent enzyme and increases the specific activity of the NADP-dependent enzyme.

Saccharomyces cerevisiae synthesizes two glutamate dehydrogenases (3, 4, 10, 11). One of these enzymes is specific for nicotinamide adenine dinucleotide (NAD) (EC 1.4.1.2; L-glutamate:NAD oxidoreductase [deaminating]); the other utilizes only NAD phosphate (NADP) (EC 1.4.1.4; L-glutamate:NADP oxidoreductase [deaminating]). In previous studies it has been demonstrated that the NAD-dependent enzyme occurs in high levels in yeast cells grown with glutamic acid as sole source of nitrogen, whereas very low amounts of the enzyme are present in cells grown with ammonia alone or glutamate and ammonia in combination (1, 2, 10, 13). On this basis it had been suggested that the NAD-dependent enzyme functions in a catabolic capacity catalyzing the breakdown of glutamate of ammonia and α-ketoglutarate. The NADP-glutamate dehydrogenase has been described as having primarily a biosynthetic function (18). Active preparations of this enzyme have been obtained from yeast cells grown with ammonia or various amino acids as the source of nitrogen (10). It has been reported that the specific activity of this enzyme is highest during the exponential phase of yeast growth on chemically defined glucose-ammonium-salts medium, with lower levels of the enzyme being observed during the stationary phase and in cells grown on complex medium (1, 20).

In spite of numerous investigations, many questions remained unanswered concerning the regulation of yeast glutamate dehydrogenases. With respect to the NADP-dependent enzyme it was unclear as to whether its synthesis was significantly affected by the nitrogen source present in the growth medium. The earlier studies by Holzer and co-workers gave some indication that the specific activity of the NADP-dependent enzyme is high in ammonia-grown cells and lower in cells grown with dicarboxylic amino acids (1). In later studies by these same workers, it seemed as if the synthesis of this enzyme is relatively independent of the state of nitrogen nutrition (10, 13). The effect of carbon or nitrogen starvation on the two glutamate dehydrogenases had not been studied in any detail by previous investigators.

The mode of regulation of yeast glutamate dehydrogenases is of particular interest to us because of the key role that these enzymes play in the interconversion of free ammonia and organic nitrogen. To understand the processes by which these enzymes are regulated, we thought that it would first be necessary to establish how various nutritional factors affect

the synthesis of the two enzymes, and then to examine in detail the molecular mechanisms which are involved. In the present communication we wish to report the results of nutritional studies concerned with yeast glutamate dehydrogenases. Our data suggest that the specific activities of both of these enzymes are influenced by the nature and by the level of the nitrogen source present in the growth medium.

MATERIALS AND METHODS

Organism. The haploid yeast strain used in these studies was *S. cerevisiae* X2180A (a SUC 2 *mal gal* 2 CUP1) obtained from the Yeast Genetics Stock Center, Donner Laboratory, University of California, Berkeley.

Growth conditions. The standard medium contained (per liter): 20 g of D-glucose, 2 g of yeast nitrogen base (Difco; no. 0335-15-9, without amino acids and ammonium sulfate), and a nitrogen source as indicated in the text. The yeast cultures were grown aerobically at 23 C with slow shaking. In most experiments the cultures were grown for 16 h from a 1% inoculum, harvested in the late exponential phase, washed with sterile distilled water, and resuspended in fresh medium to give an initial optical density (660 nm) of 1.0. This is equivalent to a cell density of approximately one-third that normally achieved in the stationary phase of growth. By using this procedure, differences in the growth phase and in the total cell mass between various cultures could be minimized. This permitted the specific activities of the glutamate dehydrogenases to be determined under relatively standard conditions regardless of the nitrogen sources used and allowed the effect of added nitrogenous compounds to be assessed after relatively short growth times. The amount of growth was routinely estimated by measuring the change in turbidity at 660 nm using cuvettes with a 10-mm path length.

Preparation of extracts. In most growth experiments, 0.5-liter samples of the yeast suspension containing 0.5 to 2.0 g of cells (wet wt) were removed at the times indicated in the text, harvested immediately (4 C) by centrifugation, washed with cold 0.1 M potassium phosphate (pH 7.5), and resuspended in 10 ml of this same solution.

The cell samples were placed in a water-jacketed processing vessel (15-ml capacity) and subjected to sonic disruption for 10 min with a Branson Sonifier, model J-17A. (Using these conditions cell breakage was in excess of 90%.) The temperature of the samples was maintained below 10 C by passing ice water through the cooling jacket. The suspension of broken cells was centrifuged for 15 min at 20,000 × *g*. The supernatant solution was carefully removed with a Pasteur pipette and stored at −20 C for assay.

Assay methods. The L-glutamate dehydrogenases were assayed spectrophotometrically at 23 C by following the oxidation of the reduced coenzyme by the method of Doherty (4). L-Asparaginase was assayed colorimetrically by measuring the formation of ammonia. The assay system contained in a final volume of 2.0 ml: 50 mM potassium phosphate buffer (pH 8.5), 25 mM L-asparagine, and enzyme protein (0.5–2.0 mg of an unfractionated extract which had been dialyzed overnight versus 100 mM potassium phosphate, pH 7.5). Samples (100 μliters) of the assay mixture were removed at 15-min intervals and immediately assayed for ammonia by nesslerization (8).

All enzyme units are reported as micromoles of substrate utilized (or product formed) per minute. Specific activities are given as units per milligram of protein. Protein concentration was determined by the biuret method (17).

RESULTS

Effect of the nitrogen source on the specific activity of glutamate dehydrogenases. The effect of various amino acids on the biosynthesis of both glutamate dehydrogenases is shown in Table 1. Extracts containing NAD-dependent enzyme of highest specific activity were obtained from cells grown on glutamate, alanine,

TABLE 1. *Effect of the nitrogen source on cell growth and the specific activity of glutamate dehydrogenases in resuspended yeast cultures*[a]

Nitrogen source	Sp act (U/mg of protein)		Cell growth[b] (%)
	NADP-GDH[c]	NAD-GDH[c]	
No nitrogen	0.03	0.003	44
$(NH_4)_2SO_4$	0.23	0.023	(100)
L-Glutamate	0.12	0.22	84
L-Aspartate	0.11	0.26	70
L-Alanine	0.14	0.25	82
L-Serine	0.14	0.043	91
L-Arginine	0.12	0.024	71
Urea	0.24	0.033	78
L-Leucine	0.10	0.082	52
L-Tryptophan	0.09	0.029	45
L-Asparagine	0.12	0.016	105
L-Glutamine	0.13	0.016	95
Allantoin	0.24	0.026	89
Casein hydrolysate	0.11		120

[a] Yeast cultures were grown for 16 h (late exponential phase) on standard medium containing 10 mM $(NH_4)_2SO_4$ as sole nitrogen source. The cells were then harvested by centrifugation and suspended in nitrogen-free growth medium to give an initial optical density (660 nm) of 1.0. Nitrogen sources were added to 0.5-liter samples of medium as indicated. Each nitrogen source was present at 20 mM except $(NH_4)_2SO_4$ (10 mM) and casein hydrolysate (0.5%). The cultures were grown aerobically at 23 C for 6 h.

[b] Growth was determined as the change in optical density (660 nm) in 6 h. All values are compared to growth on $(NH_4)_2SO_4$ which was arbitrarily set to 100%.

[c] Yeast cells were harvested and extracted, and enzyme assays were performed as given under Materials and Methods.

or aspartate. Yeast grown on most other nitrogen compounds had enzyme of significantly lower activity. The highest levels of NADP-glutamate dehydrogenase activity are found in cells grown on ammonia, allantoin, or urea, whereas growth on most α-amino acids yields extracts of lower specific activity.

Effect of carbon and nitrogen starvation on the specific activity of NAD-glutamate dehydrogenase. The specific activity of NAD-glutamate dehydrogenase rises rapidly in yeast supplied with glutamate as the sole source of nitrogen (Fig. 1). The enzyme activity reaches a maximum after 4 to 5 h and then decreases. The decrease may result from repression which occurs in response to a buildup of the endogenous pool of ammonia (1). In the absence of glucose, the specific activity of the enzyme decreases slowly. When both glutamate and ammonia are present in the medium, the specific activity of NAD-glutamate dehydrogenase decreases at a somewhat faster rate. A similar decrease is observed with cells grown on ammonia as the only nitrogen source; however, during nitrogen starvation the decrease in activity is considerably more rapid. In this experiment, during 4 h of nitrogen starvation there was a 10-fold decrease in the specific activity of NAD-glutamate dehydrogenase.

Effect of carbon and nitrogen starvation on the specific activity of NADP-glutamate dehydrogenase. NADP-glutamate dehydrogenase activity is highest when ammonium sulfate is supplied as the sole source of nitrogen (Fig. 2). Growth on glutamate or glutamate plus ammonia results in enzyme of lower specific activity. Although there is no growth and little loss of enzyme activity during carbon starvation, nitrogen starvation which permits slow growth (~50% maximal) results in a slow decrease in specific activity. This decrease is much less severe than that observed with the NAD-dependent enzyme under similar conditions. The specific activity of the NADP-dependent enzyme declines by less than 50% during 8 h of nitrogen starvation.

Effect of aspartate and glutamate on the specific activity of NADP-glutamate dehydrogenase. Figure 3 shows the effect of dicarboxylic amino acids and their amide derivatives on the level of the NADP-dependent enzyme. Cells grown on ammonia medium with these amino acids present all yield enzyme of substantially lower specific activity than that found in ammonia grown cells. (In a separate experiment it was determined that dicarboxylic amino acids were maximally effective at lowering the specific activity of the NADP-dependent enzyme when present in the growth medium at levels of 2 mM or higher. This is approximately the same level of these compounds needed to yield NAD-dependent enzyme with maximal specific activity.) In this experiment the cells were starved for nitrogen for 4 h prior to the addition of the indicated nitrogen sources to reduce the initial specific activity of the NADP-glutamate dehydrogenase. During the 6-h incubation period cell growth was approximately equivalent with ammonia alone or with ammonia plus glutamate or glutamine as nitrogen sources but was 50% reduced by the addition of

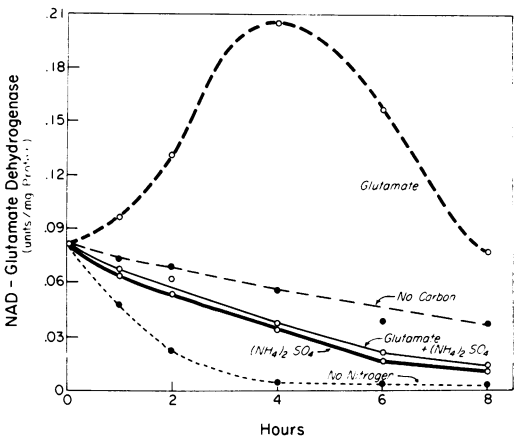

FIG. 1. *Effect of carbon and nitrogen nutrition on the specific activity of NAD-glutamate dehydrogenase. The experimental conditions were identical to those in Table 1 except that 40 mM glutamate served as the nitrogen source during the initial phase of growth. In the resuspended cultures $(NH_4)_2SO_4$ was added at a concentration of 10 mM, and L-glutamate was added at 20 mM.*

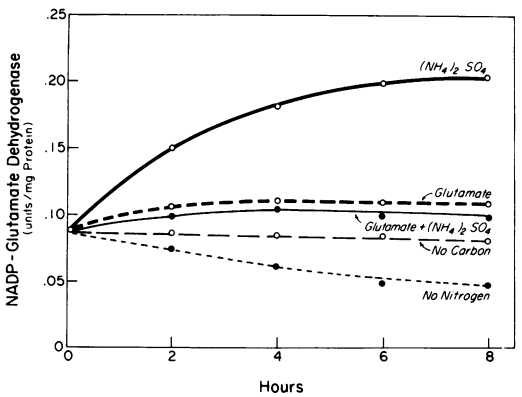

FIG. 2. *Effect of carbon and nitrogen nutrition on the specific activity of NADP-glutamate dehydrogenase. The experimental conditions were identical to those given in Fig. 1.*

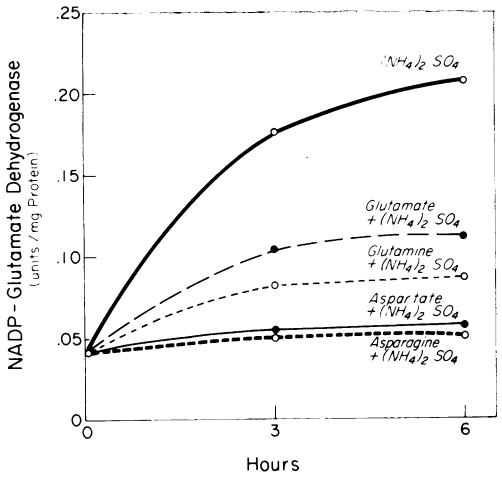

FIG. 3. *Effect of dicarboxylic amino acids and amides on the specific activity of NADP-glutamate dehydrogenase. The yeast were grown for 16 h (late exponential phase) on 4.0 liters of standard medium containing 40 mM* L*-glutamate as sole nitrogen source. The cells were harvested by centrifugation and resuspended in 10 liters of sterile nitrogen-free standard medium. After 4 h of growth, the culture was diluted with sterile nitrogen-free standard medium to give an optical density of 1.0 at 660 nm. At this time, the nitrogen sources [10 mM $(NH_4)_2SO_4$, 20 mM amino acids] were added as indicated to 1-liter samples of the cell suspensions, and growth was continued at 23 C. Samples (0.5 liter) of the grown cells were harvested after 3 and 6 h, and the enzymes were extracted and assayed as described in Materials and Methods.*

asparagine and was completely prevented by added aspartate (data not shown). Thus the low NADP-glutamate dehydrogenase activities present in aspartate- and asparagine-grown cells may represent a composite of direct repressive effects coupled with a general reduction of protein synthesis. This latter phenomenon will be reported in more detail in a subsequent communication (R. J. Roon, and H. L. Even, manuscript in preparation).

Effect of ammonia and methylamine. Figure 4 shows the decrease in NAD-glutamate dehydrogenase synthesis in response to increasing levels of ammonia (in medium containing 20 mM glutamate). The maximal negative effect on enzyme activity is achieved with 1.0 mM ammonium sulfate. Conversely, the specific activity of the NADP-dependent enzyme is very dependent on the supply of ammonia (Fig. 4). Maximal increase in activity occurs on medium containing an initial ammonium sulfate concentration of 1.1 to 1.0 mM or higher. As a control we have also determined the activity of asparaginase which has been reported to be constitutively produced in yeast (12). The specific activity of this enzyme decreases slightly in response to increased ammonia concentration in the growth medium (Fig. 4).

In previous investigations there had been no experimental evidence concerning the mechanism by which ammonia regulates glutamate dehydrogenase synthesis in yeast. The data accumulated were compatible with ammonia per se or one of its metabolic products acting in this process. In an attempt to determine whether ammonia acts directly, we measured the effect of methylamine on the cellular levels of glutamate dehydrogenases. This ammonia analogue cannot be used by yeast as a nitrogen source and is not metabolized to any detectable extent by this organism (R. Roon, unpublished data). Under the experimental conditions of Fig. 5 it does not inhibit yeast growth. However, we found that it does cause a decrease in the levels of NAD-glutamate dehydrogenase. Methylamine is most effective at a concentration of 20 mM or higher. This is approximately 10-fold higher than the concentration at which ammonia is maximally effective. Moreover, the level of enzyme activity with 20 mM methyla-

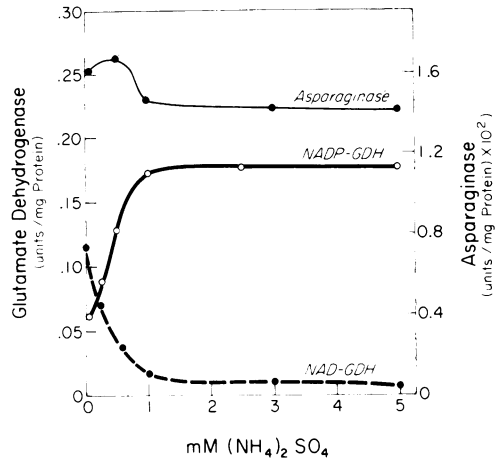

FIG. 4. *Specific activity of glutamate dehydrogenases and asparaginase as a function of the concentration of ammonium sulfate in the growth medium. Conditions for growth, enzyme extraction, and assay for the NAD-dependent enzyme were identical to those given under Table 1. Each resuspended culture received 20 mM* L*-glutamate in addition to the indicated level of $(NH_4)_2SO_4$. Samples (0.5 liter) of the resuspended cells were extracted and assayed after 3 (●--●--) h. Conditions of growth, enzyme extraction, and assay for the NADP-dependent enzyme were identical to those given under Fig. 3. The resuspended cultures received the indicated level of $(NH_4)_2SO_4$ as their sole source of nitrogen. Samples (0.5 liter) of the cell suspensions were extracted and assayed for NADP-glutamate dehydrogenase (O—O—) and for asparaginase activity (●—●—).*

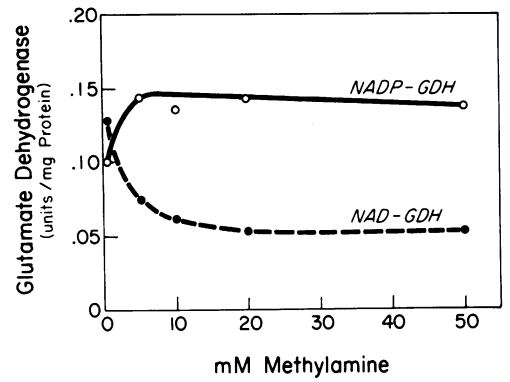

FIG. 5. *Specific activity of glutamate dehydrogenases as a function of the concentration of methylamine in the growth medium. Experimental details for the NAD-dependent enzyme were identical to those given under Table 1. Each resuspended culture received 20 mM L-glutamate in addition to the indicated level of methylamine. Samples (0.5 liter) of the cell suspensions were assayed after 3 (●--●--) h. For the NADP-dependent enzyme, experimental details were as given under Fig. 3. In addition to the indicated level of methylamine, the growth medium contained 0.25 mM ammonium sulfate. Cells were harvested, extracted, and assayed after 3 h* (O—O—).

mine is about fivefold higher than that observed in the presence of ammonia.

The effect of methylamine on NADP-glutamate dehydrogenase synthesis was also determined (Fig. 5). A stimulation of enzyme-specific activity by methylamine is observed when ammonia is supplied as growth-limiting nitrogen source at 0.25 mM. In a separate experiment, no stimulation could be detected in cells starved for nitrogen or supplied with ammonium sulfate at concentrations above 5.0 mM (data not shown).

DISCUSSION

There is probably a close relationship between the regulatory patterns which we have observed with glutamate dehydrogenases and the function of amino acids in yeast nutrition. It appears that most amino acids, including those degraded via glutamate, serve as nitrogen sources for the growth of *Saccharomyces*, but not as sole carbon or energy sources (6, 7, 21). Thus, the primary function of the two glutamate dehydrogenases seems to be to catalyze the interconversion of free ammonia and organic nitrogen. Previous investigators have suggested that the NAD-dependent enzyme serves in a catabolic capacity to provide ammonia for biosynthetic enzymes such as glutamine synthetase (1, 2, 10, 13), whereas the NADP-linked dehydrogenase is an anabolic enzyme which catalyzes the formation of glutamic acid from ammonia and α-ketoglutarate (18). The detection in the present study of reciprocal effects of ammonia and glutamate on the levels of these two enzymes provides support for this suggestion. Our experimental findings with respect to the NAD-dependent enzyme are in accord with those of Holzer and co-workers (1, 2, 10, 13), who found that this enzyme is synthesized in high levels in glutamate grown cells but is repressed in cells grown on ammonia or on glutamate plus ammonia. The sensitivity of NADP-dependent enzyme synthesis to ammonia and glutamate was not considered significant by these workers possibly because these effects are minimal in cells grown into the late exponential phase from a small inoculum. Alternately, there might be differences in sensitivity to inducers and repressors between the various yeast strains studied.

Since glutamate and other amino acids function primarily as nitrogen sources in *Saccharomyces*, it might be expected that enzymes involved in amino acid catabolism would be insensitive to catabolite repression in this organism. Similarly, enzymes active in amino acid biosynthesis should not be repressed by glucose or other readily assimilated carbon sources. Consistent with this, we have found that growth on glucose results in an increase in the specific activity of both glutamate dehydrogenases. Similar positive effects of glucose have been observed previously with arginase and ornithine transaminase in yeast (16) and with glutamate synthase and glutaminase A in *Escherichia coli* (19). The response to glucose in *E. coli* has been linked to a negative effect of cyclic adenosine monophosphate on enzyme biosynthesis.

We have found that neither glutamate dehydrogenase is derepressed by nitrogen starvation in *Saccharomyces*. In fact, the specific activity of each falls significantly. This behavior is in contrast to yeast asparaginase which remains fairly constant and to arginase and ornithine transaminase which rise in specific activity during nitrogen starvation (15, 16). The decrease in specific activity observed with the glutamate dehydrogenases can be partially accounted for if the synthesis of these enzymes is completely prevented during nitrogen starvation while the synthesis of many other proteins continues at the expense of the endogenous pool of amino acids. However, the fact that NAD-glutamate dehydrogenase activity falls much more rapidly during nitrogen starvation than during growth on ammonia suggests that some of the loss of activity may be due to an increase in the rate of enzyme degradation. This possibility is currently being investigated.

Although we have shown that exogenous

ammonia and dicarboxylic amino acids are able to affect the specific activity of both glutamate dehydrogenases, the mode of action of these compounds is not certain. Our evidence seems to be the most clearly defined with respect to the regulation of the NAD-dependent enzyme. The observation that methylamine is effective in the regulation of this enzyme suggests that ammonia per se is active in repression. It also seems likely that glutamate acts directly as an inducer of this enzyme. The only other nitrogen sources which result in its efficient synthesis are aspartate and alanine. Both of these compounds are known to be enzymatically linked with glutamate by active transaminases (14).

At present, no obvious conclusions can be drawn concerning the mode of action of ammonia and dicarboxylic amino acids in the regulation of NADP-glutamate dehydrogenase. The rather limited effect of methylamine on the synthesis of this enzyme raises some doubt concerning a direct role of ammonia in its induction. Although regulation of the NADP-dependent enzyme may involve repression by a single amino acid, the observation that growth on a number of amino acids can exert a negative effect on its specific activity suggests that a rather nonspecific response to α-amino nitrogen may be involved.

It seems clear the nature of the actual compounds involved in the regulation of yeast glutamate dehydrogenases will best be determined through the selection of appropriate nutritional and regulatory mutants having altered responses to the various effectors. At present, little work has been published concerning such mutants, with the exception of recent studies by Grenson, Wiame and co-workers (5, 9) involving a class of ammonia-resistant general amino acid permease mutants which lack the NADP-glutamate dehydrogenase. We are presently selecting mutants with altered regulation of the glutamate dehydrogenases in order to clarify the mechanism of regulation of these enzymes.

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