In vivo* Regulation of NADP-specific Glutamate Dehydrogenase by L-Amides in *Stemphylium botryosum

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The activity of NADP-specific glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4) increased at a linear rate of 2.1×10^3 units h^{-1} (g fresh wt) $^{-1}$ following the transfer of *Stemphylium botryosum* mycelium grown in L-asparagine medium to nitrate medium. The maximum enzyme activity was reached after 5 h. *De novo* synthesis was demonstrated by density labelling of the enzyme with deuterium and inhibition of NADP-GDH synthesis by cycloheximide, *p*-fluorophenylalanine or 6-methylpurine. L-Asparagine or L-glutamine could serve as a corepressor of NADP-GDH synthesis whereas the D-isomers were ineffective. Of the various amide derivatives tested, only L-asparagine *tert*-butyl ester could mimic the effect of L-asparagine. Enzyme repression was not correlated with the internal pool of L-amides. After NADP-GDH had been induced to the maximum level, the addition of L-asparagine and cycloheximide resulted in a decrease of activity with half-lives of 4.5 h and 8 h respectively. The mean half-life, as measured by following the decay in specific radioactivity of the enzyme in nitrate medium after administration of $^{35}\text{SO}_4^{2-}$, was 7 h. Mycelium starved of carbon and nitrogen sources showed a slow decrease (half-life of 17 h) in NADP-GDH activity. Depletion of energy by carbon starvation or the presence of sodium azide did not prevent the decrease in enzyme activity caused by L-asparagine. The decrease in NADP-GDH activity mediated by L-asparagine was inhibited by cycloheximide or α -iodoacetamide. Sodium azide inhibited the decrease in enzyme activity caused by cycloheximide.

INTRODUCTION

In contrast to the dual specificity of mammalian glutamate dehydrogenases (GDHs) for NAD and NADP, the enzymes from micro-organisms and plants exhibit preference for only one of the two coenzymes (Goldin & Frieden, 1971). A number of fungi contain both NAD-GDH and NADP-GDH (Goldin & Frieden, 1971; Pateman & Kinghorn, 1975), whereas others contain only NAD-GDH (Goldin & Frieden, 1971; Stevenson & Le John, 1971) or NADP-GDH (Barash & Mor, 1973). This distinct coenzyme specificity appears to be associated with two different physiological functions: the NADP-GDH being an anabolic enzyme and the NAD-GDH being a catabolic enzyme (Goldin & Frieden, 1971). The involvement of NADP-GDH in ammonia repression of several enzymes and uptake systems has been demonstrated in *Saccharomyces cerevisiae* (Grenson & Hou, 1972) and *Aspergillus nidulans* (Pateman & Kinghorn, 1975).

Pateman (1969) has presented evidence for the role of L-glutamate as a corepressor of NADP-GDH synthesis in *A. nidulans*, *Neurospora crassa* and *Escherichia coli*. The decrease in enzyme activity during growth on ammonia and amino acids was due to indirect effects on the concentration of L-glutamate in the cells (Pateman, 1969). Repression of NADP-GDH

by L-glutamate was also detected in other micro-organisms (Goldin & Frieden, 1971) but was absent in germinated spores of *Geotrichum candidum* (Barash & Mor, 1973), *Candida utilis* (Ferguson & Sims, 1974a) and *Schizophyllum commune* (Dennen & Niederpruem, 1967). Hynes (1974) showed that, in *A. nidulans*, NADP-GDH activity was rapidly lost from mycelium starved of a carbon source. Although glutamate repressed NADP-GDH synthesis, it did not cause a rapid loss in its activity (Hynes, 1974). With other fungal enzymes such as nitrate reductase (Lewis & Fincham, 1970; Subramanian & Sorger, 1972) and glutamine synthetase (Ferguson & Sims, 1974b), the addition of ammonia or L-glutamine, respectively, resulted in repression of enzyme biosynthesis and a rapid and specific inactivation of the appropriate enzyme.

Most knowledge about the control of nitrogen metabolism in fungi derives from only a few species (Pateman & Kinghorn, 1975) which are readily amenable for genetic studies. It is obvious, however, that in a large and diverse group of organisms such as fungi, considerable differences in metabolic regulation might exist. We therefore carried out experiments to investigate factors which may affect the *in vivo* regulation of NADP-GDH in the mycelial phytopathogenic fungus *Stemphylium botryosum*.

METHODS

Organism and culture conditions. A wild-type strain of *Stemphylium botryosum* Wallroth, pathogenic on lettuce, was used throughout these studies. Mycelium was grown in Roux bottles containing 100 ml of liquid medium and incubated for 5 d at 25 °C. Growth medium (no. 1) was made in 0.1 M-potassium phosphate buffer pH 6.5 and contained (mM): MgSO₄, 2; KCl, 6.7; K₂HPO₄, 1.2; FeCl₃, 0.06; sucrose, 30; asparagine, 50; and chloramphenicol, 0.15. The mycelium was removed from the growth medium by suction filtration on an Ederol filter paper (no. 15) and washed several times with deionized water by successive resuspension and filtration. To stimulate NADP-GDH production, the mycelium (0.2 to 0.4 g) was transferred to Erlenmeyer flasks (100 ml) containing 20 ml of medium no. 2. This medium was made in 0.1 M-phosphate buffer pH 6.5 and contained (mM): KCl, 6.7; MgSO₄, 2; KNO₃, 50; and sucrose, 15. The flasks were incubated for 24 h on a reciprocal shaker (140 oscillations min⁻¹) at 25 °C and the mycelium was then transferred to fresh medium (no. 2) and incubated with various nitrogenous and other compounds as described in Results. After harvest, the mycelium was carefully dried by blotting with filter paper and used for fresh weight determination.

Extraction and purification procedures. Mycelium (300 mg fresh wt) was placed in 5 ml 0.1 M-Tris/HCl buffer, pH 8.2, containing 1 mM-2-mercaptoethanol and 10 % (v/v) glycerol. All the following procedures were performed at 4 °C. The mycelium was disrupted for 30 s in a Sorval Omnimixer (model 17220) and the resulting suspension was ruptured by passage through a pre-cooled Aminco French pressure cell at 100 MPa. The cell debris was removed by centrifuging at 27000 g for 15 min; the supernatant was clarified by addition of 0.025 vol.1 M-MnCl₂, allowed to stand for 10 min and then centrifuged at 15000 g for 10 min. The NADP-GDH in the clarified extract was precipitated with ammonium sulphate between 60 and 80 % saturation. The precipitate was dissolved in 2 ml 0.05 M-Tris/HCl buffer, pH 8.2, containing 10 % (v/v) glycerol and 1 mM-2-mercaptoethanol, and then dialysed for 3 h against the same buffer. The enzyme preparation was placed on a column (0.5 × 7 cm) of DEAE-cellulose (Whatman microgranular DE-52) previously equilibrated with the above buffer and the column was washed by stepwise addition of 15 ml portions of the buffer and buffer containing 0.08 M-KCl. The enzyme was then eluted with 5 ml of similar buffer containing 0.12 M-KCl. The eluted enzyme was dialysed against 0.05 M-Tris/HCl buffer, pH 8.2, for 3 h with two changes of buffer. The dialysed enzyme preparation was then further purified by isopycnic equilibrium centrifugation.

The procedure for density gradient centrifugation was that of Filner & Varner (1967). Enzyme solution, containing 10 µg lactate dehydrogenase (EC 1.1.1.27, Sigma) as a marker, was uniformly mixed with CsCl to give a final volume of 3 ml and a density of 1.30 g ml⁻¹. Each tube contained approximately 200 units of NADP-GDH. The tubes were centrifuged at 270000 g for 65 h at 4 °C in a Beckman model L3-50 ultracentrifuge using a SW-50.1 rotor. After centrifugation, the tubes were punctured at the bottom and two-drop fractions were collected in the cold. The refractive index of every fifth fraction was determined with a Bausch and Lomb Abbe-32 refractometer, and the measured values were converted to density units using a standard curve. Lactate dehydrogenase activity was assayed according to the method of Kornberg (1955). Peak centres were determined from a plot on Gaussian paper (Ohno, 1961).

Disc electrophoresis on 7 % (w/v) acrylamide was performed according to Ornstein & Davis (1962) using Tris/glycine as a tank buffer at pH 8.3. The current was regulated to 4 mA per column. The electrophoresis

was terminated when the tracking dye, bromophenol blue, left the bottom of the column. GDH activity was detected by the tetrazolium technique (Fine & Costello, 1963) and protein by the Coomassie brilliant blue reagent (Fazekas de St Groth, Webster & Datyner, 1963). Scanner tracing of the gels was made using a Gilford 2410 transport scanner at 610 nm. For measuring radioactivity, corresponding gels were cut by a Yeda macrotoime (Rehovot, Israel) into 1 mm sections. Each section was placed in a small vial containing 0.2 ml 30 % (v/v) H_2O_2 . The vials were incubated for 18 h at 60 °C; then 3 ml Packard instagel was added to each vial and radioactivity was measured with a Packard model 3375 liquid scintillation spectrometer.

Enzyme assays. GDH [EC 1.4.1.4; L-glutamate:NADP⁺ oxidoreductase (deaminating)] was assayed by measuring the initial rate of oxidation of NADPH at 340 nm in a Gilford 2400 automatic recording spectrophotometer. The reaction mixture (total volume 2 ml) contained (mM); Tris/HCl, pH 8.2, 100; α -ketoglutarate, 30; NH_4Cl , 50; NADPH, 0.25; and 20 to 100 units of enzyme preparation. Malic dehydrogenase (MDH; EC 1.1.1.82; L-malate:NADP⁺ oxidoreductase) was measured according to Barash, Sadon & Mor (1974). All assays were carried out at 28 °C. One unit of activity was defined as the amount of enzyme causing a change in absorbance at 340 nm of 0.01 min^{-1} under the standard assay conditions. Specific activity was calculated as units (mg protein)⁻¹.

Analytical procedures. For determination of amino acids and ammonia, the harvested mycelium was washed exhaustively with distilled water and immediately freeze-dried. The free amino acids were extracted twice from 50 mg dried mycelium with 2 ml 80 % (v/v) boiling ethanol for 30 min. A sample was taken for determination of amino acids by the ninhydrin procedure (Moor & Stein, 1948) using glutamate as a standard. A Technicon automatic amino acid analyser, with norleucine as internal standard, was used to separate and quantify individual amino acids. Ammonia was extracted twice from 50 mg dried mycelium with 2 ml double-distilled water at 90 °C for 30 min, and determined by the procedure of Buttery & Rousell (1971). Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Chemicals. D_2O (99.75 %), spectroscopy grade, was purchased from Merck. $^{35}\text{SO}_4^{2-}$ (carrier free) was obtained from The Radiochemical Centre, Amersham. Asparagine and amino acid derivatives were obtained from Sigma. 5-Diazo-4-oxo-L-norvaline was a gift from R. E. Handschumacher, Department of Pharmacology, Yale University, U.S.A.

RESULTS

Biosynthesis of NADP-GDH

The kinetics of NADP-GDH synthesis following the transfer of mycelium grown on L-asparagine medium (no. 1) to nitrate medium (no. 2) are shown in Fig. 1. Enzyme activity increased at a linear rate of approximately $2.1 \times 10^3 \text{ units h}^{-1} (\text{g fresh wt})^{-1}$. The maximum activity was reached after 5 h and remained the same thereafter. Metabolic inhibitors such as cycloheximide, *p*-fluorophenylalanine and 6-methylpurine, at a concentration of 1 mM, inhibited enzyme formation by 90 % or more. These results suggest that synthesis of enzyme depends on protein and RNA synthesis. Additional evidence for *de novo* synthesis of NADP-GDH was obtained by density labelling of the enzyme. Mycelium grown in the presence of L-asparagine was incubated for 18 h in nitrate medium with either 70 % D_2O or H_2O . The enzyme was then extracted and subjected to isopycnic equilibrium centrifugation as described in Methods. The enzyme from mycelium incubated with D_2O had a buoyant density of 1.30 g ml^{-1} whereas NADP-GDH from mycelium incubated with H_2O had a buoyant density of 1.285 g ml^{-1} (Fig. 2). When mycelium containing the light enzyme was incubated with D_2O for 18 h in the presence of 10 mM-L-asparagine, no shift in the buoyant density was observed. Consequently, the possibility that deuterium exchange with pre-existing enzyme contributed to the density shift (Anstine *et al.*, 1970) can be excluded. A single band of NADP-GDH was detected when crude or purified enzyme preparations were subjected to disc electrophoresis and specifically stained for either NAD- or NADP-linked GDHs.

Repression of NADP-GDH synthesis

Mycelium of *S. botryosum* was transferred from L-asparagine medium to nitrate medium and incubated in the presence of various nitrogen sources at concentrations ranging from 0.05 to 100 mM. The cells were harvested after 18 h and their enzyme activity was determined. The results indicate that L-asparagine at a concentration as low as 1 mM caused about 60 %

Fig. 1

Fig. 2

Fig. 1. Kinetics of NADP-GDH synthesis in mycelium of *S. botryosum*. Mycelium grown on L-asparagine medium (no. 1) was transferred to nitrate medium (no. 2), as described in Methods, and enzyme activity and mycelium fresh weight were determined at various times. The mycelium was incubated without (○) and with (Δ) 1 mM-cycloheximide.

Fig. 2. Equilibrium distribution of NADP-GDH in CsCl gradients. Mycelium was incubated in nitrate medium (no. 2) for 18 h in the presence of either 70 % D₂O (Δ) or H₂O (○). LDH (□) was used as internal marker in all tubes. The LDH activities from the various tubes were superimposed. Each curve was obtained from a separate tube. Density of CsCl (●).

Table 1. *Effects of isomers or structural derivatives of asparagine and glutamine on repression of NADP-GDH*

Mycelium was incubated for 18 h in the presence of various analogues under the conditions described in the legend to Fig. 1.

Structure type	Addition (10 mM)	Repression of NADP-GDH (%) [*]
Isomers	L-Asparagine	90
	L-Glutamine	90
	D-Asparagine	0
	5-Diazo-4-oxo-L-norvaline	0
β- or γ-Carboxyl derivative	β-Aspartyl hydroxamate	23
	γ-Glutamyl hydroxamate	39
α-Amino derivatives	N-Acetylasparagine	20
	N-Acetylglutamine	0
α-Carboxyl derivative	L-Asparagine <i>tert</i> -butyl ester	85

^{*} The mean NADP-GDH activity of the control was $(120 \pm 5) \times 10^3$ units (g fresh wt)⁻¹.

repression of enzyme synthesis whereas 94% repression was obtained with 10 mM-L-asparagine (Fig. 3). L-Glutamine showed a similar effect. L-Glutamate, L-aspartate and NH₄Cl showed an effective repression only at very high concentrations (100 mM). The effect of various derivatives and isomers of asparagine or glutamine on inhibition of enzyme induction is shown in Table 1. After L-asparagine and L-glutamine, L-asparagine *tert*-butyl ester was the most effective corepressor. Hydroxamates of glutamine and asparagine caused moderate inhibition whereas other derivatives had little or no effect on enzyme synthesis.

To gain more insight into the nature of the repression process, the effects of nitrate, NH₄⁺ and L-amides on the content of the amino acid pools were studied (Table 2). The amounts of total amino acids and of ammonia increased up to 3.6-fold when a nitrogen source (5 mM) was added to nitrogen-starved mycelium. The concentration of free amino acids in the presence of external NH₄⁺ or amides (5 mM) was at least twice that in the presence of nitrate. The pools of L-glutamate, L-alanine and L-glutamine showed marked changes in response to nitrogen supply. It is noteworthy that the amount of endogenous glutamine was higher in mycelium incubated with NH₄⁺ than in the presence of the L-amides. Significant

Table 2. Effect of different nitrogen sources on the pools of amino acids and NH_4^+

Amino acids or NH_4^+ [$\mu\text{mol (g dry wt)}^{-1}$]	Nitrogen- starved mycel- ium*	Nitrogen source added (mm)†							
		KNO_3		NH_4Cl		Asparagine		Glutamine	
		0.1	5	0.1	5	0.1	5	0.1	5
Aspartate	1.73	1.8	3.1	5.5	4.0	10.5	43.0	2.4	3.4
Asparagine	1.27	1.5	1.7	6.1	3.4	11.1	92.2	1.6	2.0
Glutamate	9.17	18.5	29.5	30.2	80.1	55.3	52.0	27.0	74.7
Glutamine	2.12	4.5	5.2	4.7	36.9	10.0	9.4	6.7	25.7
Alanine	21.12	21.1	30.9	29.7	110.1	52.8	48.4	25.8	43.0
γ -Aminobutyric acid	16.69	13.8	16.2	17.7	20.5	21.4	19.1	18.4	22.1
Lysine	1.98	2.0	2.6	2.5	3.3	3.4	3.5	2.25	2.8
Arginine	5.90	5.0	8.0	6.9	10.3	10.8	14.3	8.9	9.3
Total amino acids‡	85.70	85.5	128	135	293	204	314	118	221
NH_4^+	9.20	9.7	13.5	11.4	20.7	12.5	17.1	12.7	22.3

* Mycelium was incubated on a shaker at 25 °C for 2 h in the presence of sucrose.

† Nitrogen sources were added to nitrogen-starved mycelium and incubated for 1 h.

‡ Includes additional amino acids which are not given in the Table.

Fig. 3

Fig. 4

Fig. 3. Activity of NADP-GDH in mycelium incubated with various concentrations and types of nitrogen source: ●, L-asparagine; ○, L-glutamine; □, L-glutamate; △, NH_4Cl ; ▲, L-aspartate. Mycelium was incubated for 18 h under the conditions described in the legend to Fig. 1.

Fig. 4. Effects of L-asparagine and cycloheximide on the stability of (a) MDH and (b) NADP-GDH in mycelium. Mycelium was pre-incubated for 18 h in nitrate medium (no. 2) before the addition of either 10 mM-L-asparagine (○) or 1 mM-cycloheximide (□). Activity in nitrate medium (△).

Fig. 5

Fig. 6

Fig. 5. Effect of starvation on the stability of NADP-GDH and MDH in mycelium. Mycelium was pre-incubated for 18 h in nitrate medium (no. 2), washed with 0.1 M-phosphate buffer pH 6.5, and transferred to the same buffer for further incubation. Activities of MDH (□), NADP-GDH (○) and NADP-GDH in the presence of 10 mM-L-asparagine (Δ) were measured at various times.

Fig. 6. Equilibrium distribution of purified radioactive NADP-GDH in a CsCl gradient. Enzyme preparation (200 units) from the DEAE-cellulose column was applied to each tube and purified as described in Methods. Alternate fractions were used for measuring NADP-GDH activity (●) and radioactivity (○) respectively.

amounts of L-asparagine and L-glutamine which effectively repress NADP-GDH synthesis (Fig. 3) were always present in the soluble pool (Table 2). No correlation could be detected between the pool sizes of the L-amides and enzyme repression.

Effect of L-amides on the stability of NADP-GDH in vivo

When L-asparagine was added to mycelium grown in nitrate medium, NADP-GDH activity declined rapidly whereas MDH activity remained unchanged (Fig. 4). The fall in NADP-GDH activity followed a first-order rate equation with a half-life of 4.5 h. Similar results were obtained when L-asparagine was replaced by L-glutamine. Addition of cycloheximide which effectively prevented NADP-GDH synthesis (Fig. 1), reduced the activity of both NADP-GDH and MDH. However, the half-life of NADP-GDH was considerably higher in the presence of cycloheximide (8 h) than with L-asparagine (4.5 h). Neither L-asparagine nor cycloheximide exhibited any effect on NADP-GDH activity *in vitro*.

The effect of starving mycelium of external sugars on the stability of NADP-GDH is shown in Fig. 5. Mycelium starved of nitrogen and carbon sources showed a slow decrease in NADP-GDH activity. Similar results were obtained by the removal of carbon source alone. However, sugars were not required for the effect of L-asparagine on NADP-GDH activity (Fig. 5).

Effect of metabolic inhibitors on the stability of NADP-GDH in vivo

The influence of metabolic inhibitors on NADP-GDH activity under various conditions is illustrated in Table 3. Addition of sodium azide completely prevented the decrease in enzyme activity caused by cycloheximide but did not prevent the decrease caused by L-asparagine. However, addition of α -iodoacetamide did not prevent the decrease in enzyme activity caused by cycloheximide, but did affect the decrease due to L-asparagine. The latter conclusion is based on the observation that α -iodoacetamide caused 55 to 60 % loss of NADP-GDH activity in either the presence or absence of L-asparagine as compared to 92 % loss caused by L-asparagine alone (Table 3). Enzyme activity retained with both cycloheximide and L-asparagine was higher than in the presence of the individual compounds.

Table 3. *Effect of various metabolic inhibitors on NADP-GDH activity*

Mycelium was pre-incubated for 18 h in nitrate medium (no. 2) to attain maximum enzyme activity before being transferred to the indicated media. The enzyme activity (g fresh wt^{-1}) was determined after additional incubation for 24 h.

Addition (1 mM)	NADP-GDH activity (% of control) after incubation in:		
	Nitrate medium	Nitrate medium + L-asparagine (10 mM)	Nitrate medium + cyclo- heximide (1 mM)
None	100*	8	20
Azide	90	17	90
Arsenate	90	25	10
α -Iodoacetamide	45	40	12
Cycloheximide	—	38	—

* Represents NADP-GDH activity of $(120 \pm 5) \times 10^2$ units (g fresh wt^{-1}).

Fig. 7

Fig. 8

Fig. 7. Electrophoretic separation pattern of NADP-GDH following isopycnic equilibrium centrifugation. Approximately $20 \mu\text{g}$ protein and 4 enzyme units respectively were placed on corresponding gels for detection of radioactivity ($\circ$) and enzyme activity (---).

Fig. 8. Loss of radioactivity from NADP-GDH during incubation of mycelium in nitrate medium. Experimental conditions were as described in Methods.

Determination of the half-life of NADP-GDH by an isotopic technique

The half-lives obtained by measuring the loss in enzyme activity (Fig. 4) are based on inhibition of NADP-GDH synthesis by L-asparagine and cycloheximide respectively. However, these chemicals could have stimulatory or inhibitory effects on enzyme degradation or inactivation. To determine the half-life of the enzyme in the absence of both L-asparagine and cycloheximide, mycelium grown in L-asparagine medium (no. 1) was transferred to nitrate medium (no. 2) containing MgCl_2 instead of MgSO_4 . The cells were adapted to nitrate medium in the presence of $10 \mu\text{Ci } ^{35}\text{SO}_4^{2-}$ per 20 ml culture under conditions described in Methods. After 24 h incubation, the radioactive sulphate was removed by rinsing the mycelium with nitrate medium containing MgSO_4 and further incubated in the same medium. Samples of mycelium (0.3 to 0.4 g fresh wt) were harvested at various times, frozen and stored at -15°C , until used for enzyme isolation. NADP-GDH was purified by the procedure described in Methods. The distribution of enzyme activity and radioactivity after density gradient centrifugation is shown in Fig. 6. The ratio of enzyme activity to radioactivity is not constant throughout the peak suggesting that the enzyme is not radiochemically pure. Therefore, the peak fraction alone was used for determination of the radioactivity. The purity of the enzyme in the peak fraction was tested by disc electro-

phoresis. Corresponding gels were used for detection of radioactivity, enzyme activity and protein, respectively, as described in Methods. The distribution pattern of radioactivity and enzyme activity in the acrylamide gel (Fig. 7) indicates that NADP-GDH was the only radioactive protein in the purified enzyme preparation. NADP-GDH was also the major protein band which could be detected by Coomassie brilliant blue staining. Two other protein bands appeared in trace amounts and did not contain radioactivity. After the centrifugation step, the specific activity of NADP-GDH was 12.6×10^3 units (mg protein)⁻¹, an 83-fold purification of the crude enzyme preparation. The mean half-life obtained by following the loss of radioactivity from ³⁵S-labelled enzyme was 7 h (Fig. 8).

DISCUSSION

Maximum synthesis of NADP-GDH in *S. botryosum* occurred in nitrate medium and was accompanied by a decrease in the pools of amino acids. Enzyme synthesis was repressed in media containing high concentrations of NH₄⁺ or amino acids. These observations are in accordance with the biosynthetic role assigned to NADP-GDH in other fungi (Goldin & Frieden, 1971). L-Glutamate serves as a corepressor of NADP-GDH in various fungi and bacteria (Pateman, 1969; Le John, Suzuki & Wright, 1968; Goldin & Frieden, 1971). Since the external concentration (5 mM) of L-asparagine or L-glutamine required to achieve 90 % repression was at least 20 times lower than that of NH₄⁺, L-glutamate or any other amino acid, it is reasonable to consider the L-amides rather than L-glutamate as corepressors of NADP-GDH in *S. botryosum*. L-Glutamine has been established as a corepressor of glutamine synthetase in *Candida utilis* (Ferguson & Sims, 1974*a*) and other fungi (Pateman, 1969) and an inducer of NADP-GDH in the higher plant *Lemna minor* (Rhodes, Rendon & Stewart, 1976). However, to the best of our knowledge, L-amides have not been directly implicated in repression of NADP-GDH synthesis.

The variation in NADP-GDH activity with different external nitrogen sources was not reflected by changes in the L-amide pool concentrations. Thus, although external NH₄⁺ (5 mM) and L-glutamine (5 mM) repressed enzyme synthesis by approximately 30 % and 90 %, respectively, the amount of intracellular L-amides in the presence of NH₄⁺ was higher than with glutamine. Furthermore, the L-glutamine pool concentration in the presence of NH₄⁺ was approximately 9 mM, an amount which would be expected to cause 90 % repression of NADP-GDH synthesis if given externally to the cells. The simplest hypothesis to explain the discrepancy between the effects of externally transported and internally synthesized L-amides on enzyme repression is that the L-amides are not distributed homogeneously within the fungal cells. Evidence for compartmentation of amino acids in fungi has been presented by Pateman & Kinghorn (1975). It can be postulated that the transport and channelling of L-amides to the repression site of NADP-GDH is much faster from the extracellular medium than from the intracellular pools. Such a difference in transport rates allows the cells to respond rapidly to changes in the external environment without depleting the L-amides which are vital for normal metabolism. It is noteworthy that the L-asparagine and L-glutamine enter the mycelium of *S. botryosum* by means of a common permease system (Breiman & Barash, 1976) which might be linked to the channelling of these metabolites to the repression site. Repression of enzyme by asparagine appears to be highly specific for the L-form.

Starvation or removal of the carbon source from cultures of *S. botryosum* slowed down the disappearance of NADP-GDH. In contrast, removal of the carbon source from *A. nidulans* resulted in a rapid loss of NADP-GDH activity (Hynes, 1974). L-Glutamate, which is a corepressor of this enzyme in the latter fungus, did not have any effect on enzyme disappearance in the presence of glucose. In contrast to *A. nidulans*, the inactivating or degrading system for NADP-GDH which was detected in *S. botryosum* by addition of L-asparagine was independent of glucose. The half-life for the decrease in NADP-GDH

activity in the presence of L-asparagine was 4.5 h whereas the half-life as determined by the isotopic technique was 7 h. The difference between the two values could imply that L-asparagine stimulates enzyme degradation or inactivation. However, in view of the complications involved in accurately measuring the rate constant of protein degradation by the isotopic method (Glass & Doyle, 1972; Huffaker & Peterson, 1974), the latter possibility should be taken with reservation. The disappearance of NADP-GDH was significantly slower in the presence of cycloheximide than with L-asparagine, suggesting that protein synthesis is involved in the inactivation or degradation of the enzyme. The latter point is further supported by the inhibitory effect of cycloheximide on the loss of NADP-GDH activity caused by L-asparagine. The various effects of the inhibitors on NADP-GDH are complex and may not lead to any simple interpretation. It is, however, noteworthy that azide selectively inhibited NADP-GDH disappearance due to cycloheximide but not that due to L-asparagine whereas α -iodoacetamide inhibited the L-asparagine effect. Therefore, the possibility that L-asparagine induces a specific mechanism for degradation or inactivation of NADP-GDH in *S. botryosum* cannot be excluded.

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