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GLUTAMINE SYNTHETASE AND GLUTAMATE SYNTHASE FROM *SCLEROTINIA SCLEROTIORUM*

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Key Word Index—*Sclerotinia sclerotiorum*; Sclerotiniaceae; ammonium assimilation; glutamine synthetase; transferase and biosynthetic activities; adenylylation/deadenylylation; glutamate synthase.

Abstract—Glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.13) were purified from *Sclerotinia sclerotiorum* and some of their properties studied. The GS transferase and biosynthetic activities, as well as GOGAT activity, were sensitive to feedback inhibition by amino acids and other metabolites. GS showed a marked dependence on ADP in the transferase reaction and on ATP in the Mg^{2+}-dependent biosynthetic reaction. Regulation of GS activity by adenylylation/deadenylylation was demonstrated by snake venom phosphodiesterase treatment of the purified enzyme. GOGAT required NADPH as an electron donor; NADH was inactive. GOGAT was strongly inhibited by *p*-chloromercuribenzoate and the inhibition was reversed by cysteine. The enzyme was also markedly inhibited by *o*-phenanthroline, 2,2′-bipyridyl and azaserine. L-Methionine-DL-sulphoximine (MSX) and azaserine inhibited the incorporation of ^{15}N-labelled ammonium sulphate into washed cells of *S. sclerotiorum*. MSX and azaserine respectively also inhibited purified GS and GOGAT activities. GDH activity was not detected in cell-extracts. Thus the GS/GOGAT pathway is the main route for the assimilation of ammonium compounds in this fungus.

INTRODUCTION

The assimilation of inorganic nitrogen compounds in microorganisms and plants proceeds either via glutamate dehydrogenase (GDH) or the glutamine (GS)/glutamate synthase (GOGAT) pathway. GDH has a low affinity for ammonia whereas the GS/GOGAT pathway participates in the assimilation of low concentrations of ammonia in microorganisms [1, 2]. Extensive biochemical studies have been carried out on GS in *Escherichia coli* [3]. This enzyme also functions in the assimilation of ammonium in other organisms including *Azotobacter vinelandii* [4], *Neurospora crassa* [5], *Anabaena* sp. [6] and in higher plants [7]. The glutamine synthetase from prokaryotes is regulated by feedback inhibition [8, 9], divalent cations *in vitro* [6, 10], and by an adenylylation and deadenylylation mechanism [4, 11–15].

Tempest *et al.* [2] and Brown *et al.* [16] claimed that eukaryotic organisms including *Saccharomyces cerevisiae*, *Candida utilis*, *Aspergillus nidulans* and *N. crassa* do not contain GOGAT. Subsequently, however, the enzyme was detected in some species of *Schizosaccharomyces* and *Saccharomycodes ludwigii* [16, 17] and in *S. cerevisiae* [18]. More recently GOGAT has been purified from *S. cerevisiae* [19], *N. crassa* [20] and from higher plants [21–23]. Although some properties of the enzyme and its regulation have been studied in bacteria [24–28], green algae [29] and in higher plants [21–23], there is little information for the enzyme in filamentous fungi.

In this paper we report on the purification and properties including regulation of glutamine synthetase and NADPH-glutamate synthase from *Sclerotinia sclerotiorum*.

RESULTS

Purification and some properties of GS

The enzyme was purified about 108-fold with a yield of 55% by DEAE-cellulose chromatography followed by affinity chromatography on a Blue Sepharose CL-6B column (Table 1). The transferase activity of the purified enzyme (fraction 4, Table 1) was 14 μmoles γ-glutamylhydroxamate produced/min/mg protein. The approximate M_r of the enzyme was 490 000 while the subunit M_r, determined by polyacrylamide gel electrophoresis in the presence of SDS, was 60 000. Thus the enzyme is composed of 8 subunits. The K_m value for hydroxylamine, glutamine and ADP respectively for the transferase activity (fraction 4, Table 1) calculated from double-reciprocal plots were 2.2, 4.5 and 0.14 mM.

Ammonium chloride and glutamate, the substrates for the biosynthetic reaction, competitively inhibited transferase activity with respect to glutamine (Figs 1 and 2). At 20, 10 and 5 mM glutamine respectively, glutamate (20 mM) inhibited the transferase activity by 12, 23 and 28% while ammonium chloride at the same concentration inhibited by 6, 14 and 26%.

The effects of various divalent cations at 0.5 and 5 mM on transferase activity were investigated. Maximum transferase activity was observed with Mn^{2+} at 5 mM. At this concentration low activities were recorded for Co^{2+} (19% of that for Mn^{2+}), Cu^{2+} (9%) and Mg^{2+} (2%) whereas Ni^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} were without effect. At lower concentration (0.5 mM) the highest activity was also observed with Mn^{2+} followed by Co^{2+} (24% of that for Mn^{2+}), Cu^{2+} (17%), Mg^{2+} (14%), Ni^{2+} (13%), Ca^{2+}

Table 1. Purification of glutamine synthetase

Fractions	Activity (unit)	Protein (mg)	Specific activity (unit/mg protein)	Purification (-fold)	Recovery (%)
Crude extract (S_{20})	20.80	156.80	0.13	1	100
First Blue Sepharose column	18.00	12.60	1.43	11	87
DEAE-cellulose column	16.20	8.40	1.93	15	78
Second Blue Sepharose column	11.48	0.82	14.00	108	55

One unit corresponds to 1 μmole γ-glutamylhydroxamate formed per min in the Mn^{2+}-dependent transferase assay.

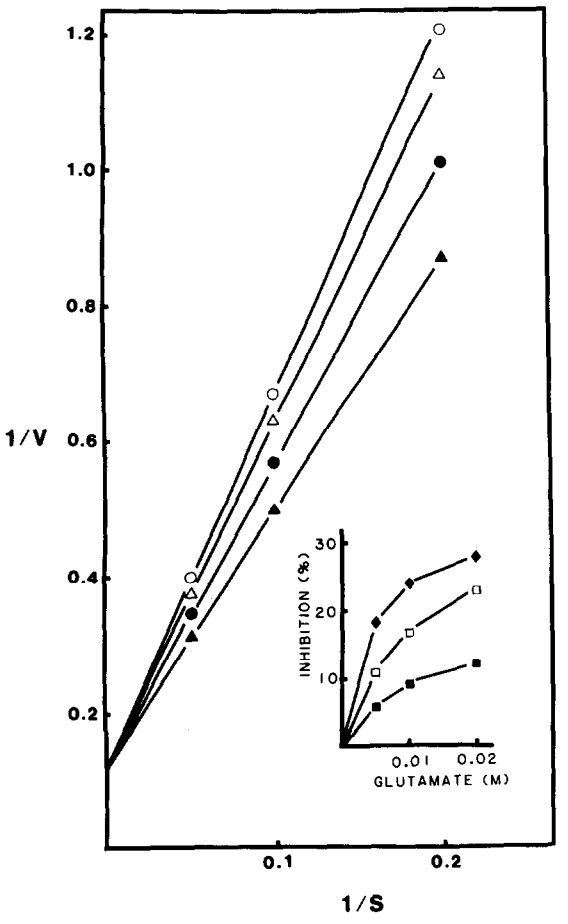

Fig. 1. Double reciprocal plots of the effects of glutamate on GS transferase activity at various concentration of glutamine. Concentration of glutamate: 0 mM, ▲; 5 mM, ●; 10 mM, △ and 20 mM, ○. Inset: Effect of glutamate with glutamine at 5 mM, ◆; 10 mM, □; and 20 mM, ■. V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein); S = substrate (glutamine, mM).

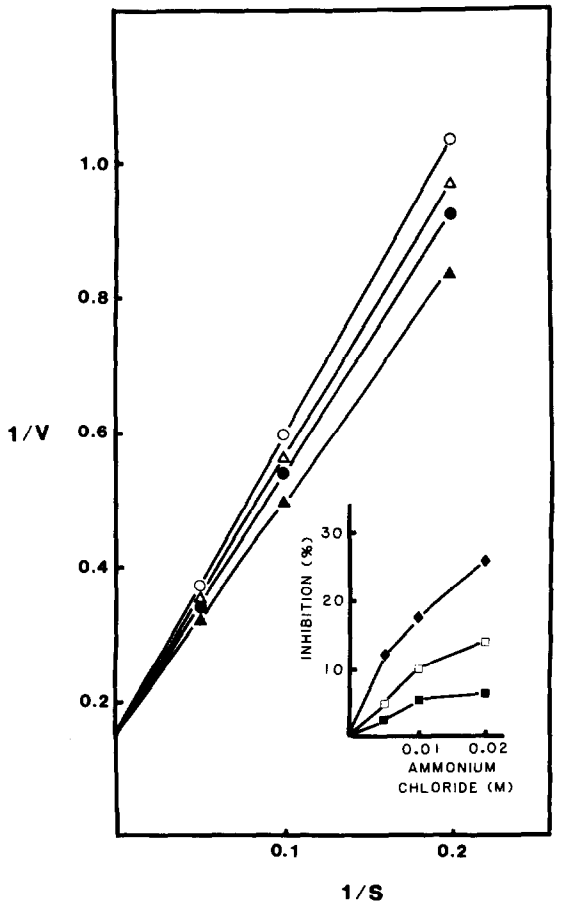

Fig. 2. Double reciprocal plots of the effects of ammonium chloride on GS transferase activity at various concentration of glutamine. Concentration of ammonium chloride: 0 mM, ▲; 5 mM, ●; 10 mM, △ and 20 mM, ○. Inset: Effect of ammonium chloride with glutamine at 5 mM, ◆; 10 mM, □ and 20 mM, ■. V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein); S = substrate (glutamine, mM).

(12%), Fe^{2+} (11%) and Zn^{2+} (10%). In contrast to the transferase enzyme, maximum activity for biosynthetic activity was obtained with Mg^{2+} at 50 mM. The order of effectiveness of the different cations was $Mg^{2+} > Co^{2+} > Mn^{2+} > Cu^{2+} > Fe^{2+} > Zn^{2+} > Ni^{2+} > Ca^{2+}$.

The effects of various concentrations of L-methionine-DL-sulphoximine (MSX), an analogue of glutamine, on enzyme activity was also studied. Thus MSX at 20 μM

inhibited transferase activity by 93% and at 1 μM by 27%.

Effects of amino acids, nucleotides and organic acids

Studies with the purified enzyme (fraction 4, Table 1) indicated that cysteine, alanine, glycine, serine, isoleucine, threonine, proline, phenylalanine and valine each at 10 mM markedly inhibited transferase activity whereas cysteine and tryptophan restricted biosynthetic activity (Table 2).

The effects of nucleotide triphosphates and diphosphates are shown in Table 3. Maximum transferase activity was recorded with ADP and lower rates with CDP, GDP, IDP and UDP whereas nucleotide triphosphates were relatively inactive. For the Mg^{2+}-dependent biosynthetic reaction ATP was the most effective nucleotide, whereas CTP, GTP or ITP substituted more effectively for ATP in the Mn^{2+}-dependent biosynthetic reaction; 65, 62 and 54%, respectively, of that with ATP.

The effects of some organic acids on transferase and biosynthetic activities are shown in Table 4. Oxalate (10 mM) markedly inhibited transferase activity by 81% and oxalacetate and α-ketoglutarate by 69 and 40%, respectively, whereas malate, lactate and pyruvate were without effect. The biosynthetic activity was inhibited by oxalate, α-ketoglutarate and oxalacetate (each at 10 mM) by 49, 49 and 42% respectively, whereas malate, lactate, citrate, pyruvate and succinate had no effect.

Table 2. Effects of various amino acids on transferase and biosynthetic activities.

Amino acids (10 mM final concentration)	Inhibition (%)	
	Transferase activity	Biosynthetic activity
Cysteine	94	80
Alanine	89	40
Glycine	78	48
Serine	74	42
Isoleucine	71	62
Threonine	70	40
Proline	66	58
Phenylalanine	66	52
Valine	63	49
Lysine	47	30
Histidine	42	52
Methionine	41	28
Asparagine	41	32
Tryptophan	33	60
Leucine	26	40
Arginine	13	16

The results are expressed as % inhibition of the control (without amino acid). Activity of the control was 14.92 μmoles γ-glutamylhydroxamate produced/min/mg protein (for transferase) and 1.23 μmole Pi produced/min/mg protein (for biosynthetic assay).

Adenylylation and deadenylylation

In some bacteria [15, 30, 31] the extent of transferase activity in the presence of 60 mM $MgCl_2$ has been used as an indication of the degree of adenylylation of the enzyme. The fully adenylylated enzyme is relatively inactive in the presence of Mg^{2+}, whereas the deadenylylated enzyme is not affected. The effects of snake venom phosphodiesterase treatment on the transferase activity of the purified enzyme (fraction 4, Table 1) in the presence and absence of Mg^{2+} are shown in Table 5. The percentage of adenylylation was reduced from 74% to 25% on treating the purified enzyme with snake venom phosphodiesterase and the A_{260} values were decreased by about 20%. More

Table 3. Effects of various nucleotides on transferase and biosynthetic activities determined as described in Experimental

Nucleotide	Activity of the control (%)		
	Transferase	Biosynthetic	
		Mg^{2+}-dependent	Mn^{2+}-dependent
ATP	5	100	100
CTP	3	8	65
GTP	4	10	62
ITP	1	6	54
UTP	3	5	25
ADP	100	4	4
CDP	42	2	2
GDP	38	5	2
IDP	42	0	3
UDP	29	0	0

Nucleotide concentrations were 0.4 mM for transferase assay and 7.5 mM for biosynthetic assay. The enzyme activity of control for transferase activity (with ADP) was 14.92 μmoles γ-glutamyl hydroxamate produced/min/mg protein, for Mg^{2+}-dependent and Mn^{2+}-dependent biosynthetic activity were 1.23 μmole and 0.95 μmole Pi produced/min/mg protein, respectively

Table 4. Effects of organic acids on transferase and biosynthetic activity

Organic acids (10 mM final concn)	Inhibition (%)	
	Transferase	Biosynthetic
Oxalate	81	49
Oxalacetate	69	42
α-Ketoglutarate	40	49
Citrate	5	0
Succinate	3	0

The results are expressed as % inhibition of the control (without organic acid). Activity of the control was 14.92 μmoles γ-glutamylhydroxamate produced/min/mg protein (for transferase activity) and 1.23 μmole Pi produced/min/mg protein (for biosynthetic activity).

direct evidence for the cleavage of AMP from the adenylylated enzyme was achieved by separating the SVD-treated enzyme by polyacrylamide (12.5%, w/v) slab gel electrophoresis. The buffer used was 0.09 M Tris, 0.08 M boric acid and 0.93 g/l Na_2-EDTA (pH 8.4), for 15 min at 20 mA then at 30 mA for 2.5 hr at 25°. The AMP was detected in the gels by UV light with reference to appropriate standards.

Purification and some properties of GOGAT

Glutamate synthase was purified about 102-fold with a yield of 38%. The purification procedure involved $MnCl_2$ precipitation, ammonium sulphate fractionation followed by separation on a DEAE-cellulose column and then by affinity chromatography on a Blue Sepharose CL-6B column (Table 6). The partially purified enzyme (fraction 4, Table 6) had a specific activity of 7.6 μmoles NADPH oxidized/min/mg protein. The K_m values for glutamine, α-ketoglutarate and NADPH respectively calculated from double reciprocal plots were 2.6 mM, 0.35 mM and 35 μM.

The data in Table 7 show that the enzyme was markedly inhibited by phenylalanine (78% at 10 mM) and to a lesser extent (< 45%) by arginine, leucine, valine and glutamate, but was not affected by histidine and asparagine. Oxalate and malate (5 mM) inhibited enzyme activity by 48 and 40%, respectively. ATP (at 5 mM) restricted activity by 47% but there was no effect at 1 mM (Table 8).

p-Chloromercuribenzoate (at 0.1 mM) and *o*-phenanthroline (at 10 mM) completely inhibited GOGAT activity; the inhibition by *p*-CMB was reversed by the addition of cysteine. This enzyme was also inhibited by 2,2'-bipyridyl (88% at 10 mM) and arsenite (57%) (Table 9). Azaserine inhibited enzyme activity, at 1 mM the inhibition was 68%.

^{15}N incorporation from $(^{15}NH_4)_2SO_4$ and effects of inhibitors

In the absence of inhibitor the amount of ^{15}N-$(NH_4)_2SO_4$ incorporated into washed cells after 1 hr incubation was 4.4 μg/mg protein. Both MSX (2 mM) and azaserine (1 mM) inhibited the incorporation of the

Table 5. Snake venom phosphodiesterase (500 μg) was added to 10 ml of a purified preparation of glutamine synthetase (fraction 4, Table 1)

Treatment	A_{260}	Transferase activity (units/mg protein)		Adenylylation (%)
		$-Mg^{2+}$	$+Mg^{2+}$	
1. Without SVD (Fraction 4, Table 1)	0.642	14.92	3.94	74
2. With SVD (G-10 fraction)	0.541	14.75	11.04	25

After incubation for 1 hr at 37° the reaction mixture was loaded onto a Sephadex G-10 column to separate AMP which was cleaved from the adenylylated enzyme. The absorbance of the enzyme at 260 nm and the transferase activity of the SVD-treated and untreated enzyme was determined.

Table 6. Purification of glutamate synthase

Fraction	Activity (unit)	Protein (mg)	Specific activity (unit/mg protein)	Purification (-fold)	Recovery (%)
Crude extract (S_{20})	12.30	164.0	0.075	1	100
$(NH_4)_2SO_4$ 25–65% fraction	12.78	37.2	0.344	5	103
DEAE-cellulose column	7.03	5.8	1.216	16	57
Blue Sepharose column	4.66	0.6	7.618	102	38

One unit corresponds to 1 μmole NADPH oxidized per min.

Table 7. Effects of various concentrations of amino acids on GOGAT activity. The results are expressed as % inhibitor of control (without amino acid)

Amino acids	Inhibition (%) 1 mM	5 mM	10 mM
Phenylalanine	14	28	78
Arginine	9	9	43
Leucine	4	5	36
Valine	0	6	26
Glutamate	9	16	25
Isoleucine	0	22	23
Aspartate	0	20	22
Serine	6	14	21
Tryptophan	5	5	20
Threonine	0	5	19
Methionine	8	13	16
Cysteine	9	9	14
Lysine	8	8	14

Activity of the control was 7.6 μmoles NADPH oxidized/min/mg protein.

Table 8. Effects of various metabolites at different concentration on GOGAT activity

Metabolites	Inhibition (%) 1 mM	5 mM
c-AMP	0	5
AMP	0	31
ADP	18	29
ATP	0	47
Pyruvate	14	20
Citrate	8	16
Succinate	9	14
Fumarate	16	29
Malate	11	40
Oxalacetate	15	20
Oxalate	26	48

The results are expressed as % inhibition of control (without metabolite). Activity of the control was 7.6 μmoles NADPH oxidized/min/mg protein. The enzyme activity was determined as described in Experimental.

labelled compound into cell-nitrogen by 52 and 31% respectively.

DISCUSSION

A range of microorganisms and plants reduce nitrate to ammonia which is then incorporated into amino nitrogen compounds. It is established that glutamine synthetase/glutamate synthase pathway is a major route whereby ammonia is assimilated when low amounts of ammonia are present. The results presented herein for *S. sclerotiorum* grown with nitrate are in accord with this concept.

Table 9. Effects of various concentrations of inhibitors on GOGAT activity

Inhibitors	Concentration (mM)	Inhibition (%)
Sodium arsenite	4	9
	10	57
Sodium azide	10	5
	25	15
Potassium cyanide	10	4
	25	94
2,2'-bipyridyl	1	4
	5	43
	10	88
o-Phenanthroline	1	8
	4	68
	10	100
p-CMB	0.01	46
	0.05	96
	0.10	100
p-CMB + Cysteine	0.1 + 0.2	0
Azaserine	0.2	14
	0.6	42
	1.0	68

The enzyme was preincubated for 5 min with the inhibitor before initiating the reaction. The results are expressed as % inhibition of control (without inhibitor). The activity of the control was 7.6 μmoles NADPH oxidized/min/mg protein.

Purified GS from the fungus which had an estimated M_r of 490 000 was composed of eight subunits each with a M_r of 60 000. These results are similar to the subunit values for GS from *Rhizobium japonicum* strain CC705 and CC723 [32] and from *Methylococcus capsulatus* [12]. The K_m values for the purified enzyme were 2.2 mM for hydroxylamine, 4.5 mM for glutamine and 0.14 mM for ADP. These values are lower than those for the enzyme from *M. capsulatus* [12] and from *Chlorobium vibrioforme* f. *thiosulfatophilum* [8].

The inhibition of transferase activity by glutamate and NH_4Cl (the substrates for biosynthetic reaction) was competitive for glutamine, in agreement with the data for *Anabaena cylindrica* [33], *Ch. vibrioforme* f. *thiosulfatophilum* [9] and *Rh. japonicum* [13]. This result indicates that glutamine interacts with the enzyme, so that its NH_2 group occupies the ammonia binding site while the oxygen binding site, to which glutamate is bound, is required for the attachment of the oxygen atom of glutamine as postulated by Gass and Meister [34]. The specific dependence of the transferase activity for Mn^{2+} is in agreement with the data for the enzyme from *A. cylindrica* [33], *Rh. japonicum* [32] and *M. capsulatus* [12].

Purified enzyme from the fungus was sensitive to various feedback inhibitors in accord with the results for the enzyme from other sources. Thus alanine, glycine and serine inhibited the enzyme from *Anabaena* L-31 [8], *Anabaena flos-agua* [35], *Rhodopseudomonas palustris* [30] and *Ch. vibrioforme* f. *thiosulfatophilum* [9] and in *S. sclerotiorum*. In addition cysteine, isoleucine, threonine, phenylalanine and valine restricted the enzyme. The inhibition of the transferase activity by oxalate, oxalacetate and α-ketoglutarate is also in agreement with data

for the enzyme from *Bacillus stearothermophilus* [36], *Nitrosomonas europaea* [37] and *Rh. japonicum* [32]. The fungal enzyme showed a marked dependence on ATP in the Mg^{2+}-dependent biosynthetic activity in agreement with the data for *Anabaena* sp. [6]. This result is somewhat unusual in that the enzyme from *E. coli* [38] and *M. capsulatus* [12] has been shown to utilize other nucleotides, especially GTP, in the presence of Mg^{2+}. When Mn^{2+} was used instead of Mg^{2+} then other nucleotide triphosphates functioned in the biosynthetic reaction. The transferase activity was maximal with ADP and other nucleotide diphosphates had lower activities in agreement with the enzyme from *E. coli* [38] and *M. capsulatus* [12].

GS from *S. sclerotiorum* grown with nitrate was partially adenylylated in agreement with the result for a range of microorganisms. Since the adenylylated form of GS could be deadenylylated by SVD, the enzyme from this fungus is regulated by a adenylylation/deadenylylation mechanism.

GOGAT exists in eukaryotes (including higher plants) in two forms, one utilizing NAD(P)H as a reductant [18, 20, 39, 40]; the other reduced ferredoxin [22, 29, 41]. In contrast to the enzyme from *N. crassa* [20] and *S. cerevisiae* [18] which is NADH-dependent, GOGAT in *S. sclerotiorum* requires NADPH. The K_m values for glutamine (2.6 mM), α-ketoglutarate (0.35 mM) and NADPH (35 μM) were slightly higher than those for the enzyme from *S. cerevisiae* [18], *Thiobacillus thioparus* [27] and *Ch. vibrioforme* f. *thiosulfatophilum* [9]. Of the 19 amino acids tested, phenylalanine (10 mM) inhibited GOGAT activity by more than half whereas only serine at the same concentration restricted the enzyme from *Th. thioparus* by 44% [27]. The enzyme from *S. sclerotiorum* was inhibited 47% by ATP (5 mM) whereas the nucleotide at a similar concentration inhibited GOGAT activity from *Th. thioparus* by 8% only.

The enzyme was completely inhibited by *p*-CMB at 0.1 mM; this effect was reversed by the addition of cysteine (0.2 mM). This result indicating the importance of thiol groups for the enzyme activity is in accord to the result of Adachi and Suzuki [27] for the enzyme from *Th. thioparus*. The enzyme from the fungus was also inhibited completely by *o*-phenanthroline (10 mM) and by 2,2′-bipyridyl (88%).

In the present work the incorporation of ^{15}N-labelled $(NH_4)_2SO_4$ into washed cells of *S. sclerotiorum* was markedly inhibited by MSX, an inhibitor of GS, and to a lesser extent by azaserine, an inhibitor of GOGAT. The inhibitory effects of MSX and azaserine were also observed in purified GS and GOGAT respectively. No GDH activity was detected in cell extracts. Thus the GS/GOGAT pathway is the main route for the assimilation of ammonia in *S. sclerotiorum* grown with nitrate.

EXPERIMENTAL

Growth conditions. Sclerotinia sclerotiorum was kindly supplied by Dr. M. Carter, Dept. of Plant Pathology of this Institute. The fungus was maintained on Czapek-Dox agar plates containing per liter: glucose, 15 g; $NaNO_3$, 2 g; KH_2PO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; $MnSO_4 \cdot H_2O$, 0.42 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.40 mg and agar (for solid medium), 20 g. One square cm mycelial discs from the outermost growth zone of 4–5 day-old cultures on agar plates grown at 25° were used as inocula. Into 100 ml sterile cultures in 250 ml flasks stoppered with cotton wool was added three discs of inocula. After incubating at 30° on a gyratory shaker for 4 days the contents of each flask was transferred into 1 l. flasks containing 300 ml sterile culture medium and incubation continued for a further 2 days. The felts harvested by centrifugation at 9000 *g* for 5 min at 4° in a RC-2B refrigerated centrifuge were washed three times with cold Tris–HCl buffer (pH 7.0) and then blotted between towel paper to remove water. The blotted mycelial pads could be stored at −15° for 1 month without loss of enzyme activity.

Preparation of crude extracts. Preparation of cell extracts and subsequent purification of the enzymes were carried out at 4°. Frozen mycelial pads were ground in a cold porcelain pestle and mortar using either 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ (for GS) or 50 mM Tris–HCl buffer (pH 7.5) for GOGAT and then passed × 3 through a French pressure cell at 20 000 psi. The homogenate was centrifuged at 20 000 *g* for 20 min and the supernatant (S_{20}) used as a crude extract.

Enzyme assays. Glutamine synthetase was assayed by the γ-glutamyltransferase reaction according to the procedure of Shapiro and Stadtman [42] except that 0.3 mM $MnCl_2$ was used. One unit of transferase activity is defined as 1 μmole γ-glutamylhydroxamate produced per min. Biosynthetic activity was measured as described by Shapiro and Stadtman [42] except that the final volume of reaction mixture was 0.5 ml and the reaction was terminated with 3 ml $FeSO_4$ followed by 0.3 ml $(NH_4)_6Mo_7O_{24}$ reagents. One unit of biosynthetic activity is defined as 1 μmole Pi produced per min.

Glutamate synthase was assayed at 30° by measuring the initial rate of oxidation of NADPH in a 1 cm quartz cuvette at 340 nm in a recording spectrophotometer (Varian Techtron model 635, Melbourne, Australia). The reaction mixture, in 3 ml final volume, contained 6 mM glutamine, 3 mM α-ketoglutarate, 0.1 mM NADPH and 100 mM Na-phosphate buffer (pH 8.0). The reaction was started by adding α-ketoglutarate. One unit of enzyme activity is defined as 1 μmole NADPH oxidized per min.

Adenylylation state of GS. To determine the extent of adenylylation, GS was assayed with and without 60 mM $MgCl_2$ and the values obtained were then used in the formula proposed by Shapiro and Stadtman [42]. The effect of snake venom phosphodiesterase on the deadenylylation of the enzyme was also determined by this method.

Protein content. Protein was determined by the method of Bradford [43] using bovine serum albumin as a protein standard.

Purification of GS. Glutamine synthetase was purified by the procedure described below. All operations were carried at 4°. The crude extract (S_{20}) was adsorbed onto a Blue Sepharose CL-6B column (115 × 15 mm) equilibrated with 50 mM Tris–HCl (pH 7.0) containing 1 mM $MnCl_2$ (the buffer). The column was washed with approximately 200 ml of the buffer before elution with the buffer containing 2 mM ADP. Active fractions from the column were pooled and then loaded onto a DEAE-cellulose (DE-52) column (200 × 25 mm) pre-equilibrated with the buffer. After loading the enzyme, the column was washed with approximately 250 ml of the buffer. GS was separated by a linear gradient from 0 to 500 mM NaCl in the same buffer. The total gradient volume was 150 ml and the flow rate was 50 ml/hr. Active fractions were pooled and dialysed overnight against 3 l. of 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM $MnCl_2$. The dialysed solution was subjected to a second Blue Sepharose CL-6B chromatography as described above. Details of the purification are presented in Table 1.

Purification of GOGAT. Glutamate synthase was partially purified by the procedure given below. All the operations were carried out at 4°. To the crude extract, $MnCl_2$ (32 ml of a 1 M soln per l. of crude extract) was added slowly with constant stirring.

The mixture was then centrifuged at 20000 *g* for 15 min and the supernatant was collected. Solid $(NH_4)_2SO_4$ was gradually added to the supernatant with constant stirring to produce 25% (w/v) $(NH_4)_2SO_4$ saturation. During this addition, pH was maintained at 7.5 by adding 50 mM Tris. After standing for 30 min the mixture was centrifuged at 20000 *g* for 15 min. The supernatant was then brought to 65% (w/v) $(NH_4)_2SO_4$ saturation and allowed to stand for 30 min before centrifuging at 20000 *g* for 15 min. The pellet redissolved in 50 mM Tris–HCl (pH 7.5) was recentrifuged at 20000 *g* for 10 min to remove insoluble materials.

The clarified soln dialysed overnight against 3 l. of 50 mM Tris–HCl buffer (pH 7.5) was then loaded onto a DE-32 column (140 × 35 mM) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The column was then washed with 300 ml of the same buffer. GOGAT was separated by a linear gradient of NaCl (0–500 mM) in the same buffer. The total gradient volume was 250 ml and the flow rate was 50 ml/hr. Active fractions were pooled and dialysed overnight against 3 l. of the same buffer.

The dialysed enzyme was loaded onto a Blue Sepharose CL-6B column (80 × 15 mM) which had been pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5) and then washed with approximately 150 ml of the same buffer. The enzyme was eluted with 20 ml of the same buffer containing 1 mM NADPH. A purified enzyme (102-fold) with a recovery of 38% was obtained by this procedure (Table 6).

Molecular weight determinations. The M_r of the purified GS was determined by means of gel filtration using a Sepharose 6B column pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ according to the method of Andrews [44]. The following proteins dissolved in the equilibration buffer were used as markers: thyroglobulin (669000), ferritin (440000), catalase (232000) and aldolase (158000).

The M_r of the enzyme subunit was determined by polyacrylamide gel electrophoresis (12.5% (w/v) acrylamide) in the presence of 0.1% (w/v) sodium dodecyl sulphate. The protein soln was boiled for 3 min in the presence of 1% (w/v) SDS and 5% (w/v) β-mercaptoethanol. The proteins used as markers were phosphorylase *b* (subunit 94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20000) and L-lactalbumin (14400).

$^{15}NH_4^+$ incorporation into washed cells. Washed felts (*ca* 1 g fr. wt) were suspended with gentle shaking into 20 ml of N-free culture medium. The suspensions were incubated with and without MSX (2 mM) or azaserine (1 mM) for 2 hr at 30°. Then into each sample was added $(^{15}NH_4)_2SO_4$ (5 mM final concentration) and incubation continued for a further 1 hr. The cells, filtered through Whatman paper No. 541 in a glass funnel connected to a vacuum flask, were washed thoroughly with cold glass distilled water. The cells were then dispensed into micro-Kjeldahl flasks for digestion. Samples for ^{15}N enrichment analysis were prepared as described by Brownell and Nicholas [45]. Measurements of ^{15}N enrichment were carried out in a 602E Mass spectrometer (ISOMASS, Middlewich, Cheshire, UK).

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