Metabolism of L-Threonine and its Relationship to Sclerotium Formation in *Sclerotium rolfii*

By G. KRITZMAN, Y. OKON, I. CHET AND Y. HENIS

*Department of Plant Pathology and Microbiology,
The Hebrew University of Jerusalem, Rehovot, Israel*

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SUMMARY

The activities of L-threonine dehydrogenase (I), 2-amino-3-oxybutyrate:CoA ligase (II), malate synthetase (III), isocitrate lyase (IV), glyoxylate dehydrogenase (V), glycine decarboxylase (VI), L-serine hydroxymethyltransferase (VII), glucan synthetase (VIII), glucose 6-phosphate dehydrogenase (IX) and succinic dehydrogenase (X) were detected in cell-free extracts prepared from the mycelium of the fungus *Sclerotium rolfii* type R. Transfer of *S. rolfii* to a threonine-containing medium resulted in a significant increase in the intracellular concentrations of L-threonine, glycine, serine and glyoxylate, and a decrease in oxalate. Incubation with ^{14}C -labelled L-threonine resulted in an immediate output of $^{14}\text{CO}_2$, and an accumulation of labelled glycine and serine in the mycelium. L-Threonine (10^{-2} M) increased branching, favoured formation of sclerotia, and induced the formation of enzymes I to VIII, but not IX and X. Sodium oxalate (1.5×10^{-2} M) inhibited branching, sclerotium formation and the activity of enzymes III and IV. Glycine (10^{-1} M) inhibited branching, sclerotium formation and activity of I and II. Ammonium chloride (10^{-1} to 10^{-3} M) inhibited formation of sclerotia, threonine uptake and activity of III. Acetyl-CoA inhibited V and L-cysteine inhibited I as well as sclerotium formation and branching. It is suggested that hyphal morphogenesis and formation of sclerotia in *S. rolfii* require an increased supply of carbohydrate intermediates and energy and that these are mainly supplied by the glyoxylate pathway.

INTRODUCTION

The effect of amino acids on morphogenesis has been observed in several fungi, and includes control of lateral hypha frequency in *Mucor hiemalis* (Plunkett, 1966) and sclerotium formation in *Sclerotium rolfii* (Chet, Henis & Mitchell, 1966; Henis, Okon & Chet, 1973). Liu & Wu (1971) reported that L-threonine induced sclerotium formation in *S. rolfii*, but made no attempt to explain the mechanism of this effect. The metabolism of L-threonine, given as the sole carbon and nitrogen source, was studied in detail in penicillia and fusaria by Willetts (1972*a, b*) and by Willetts & Turner (1971) who showed that in these fungal groups, L-threonine could be metabolized via the glyoxylate pathway. Although the presence of this metabolic pathway in *S. rolfii* has been demonstrated (Maxwell & Bateman, 1968*a*), its role in L-threonine metabolism in this fungus has not yet been studied.

The purpose of this work was to reveal the possible links between the effect of L-threonine on the morphogenesis of *S. rolfii* and its metabolic pathway in this fungus.

METHODS

Strain and growth conditions. *Sclerotium rolfii* Sacc. type R ATCC26326 (Chet & Henis, 1972) was grown at 30 °C on a cellophane membrane in Petri dishes (8.5 cm diam.) containing 15 ml of synthetic medium (SM) (Okon, Chet & Henis, 1973). The plates were inoculated in the centre with agar discs (0.5 cm diam.) covered with fungal mycelium which had been cut from a 5-day-old colony. The following supplements (AR grade, final concentration) were added separately to the growth medium: L-threonine, 10^{-2} M; glycine, 10^{-1} M; sodium acetate, 0.2 M; sodium oxalate, 1.5×10^{-2} M; L-cysteine, 10^{-2} M; NH_4NO_3 , 0.01 to 0.02 M; NH_4Cl , 0.01 to 0.2 M; and NaNO_3 , 0.01 to 0.2 M. Ten-fold concentrated solutions of these supplements were sterilized by filtration through a 0.45 μm HA Millipore filter and added aseptically to the melted agar.

The effect of these supplements as well as of mitomycin C, DL-*p*-fluorophenylalanine and cycloheximide (Sigma) on morphogenesis, sclerotium formation and enzyme composition was studied by transferring colonies grown on cellophane from a non-supplemented medium to a supplemented one. No degradation of the cellophane was observed when *S. rolfii* was grown in the presence of glucose during 10 days of incubation.

L-Threonine uptake and transport. The uptake and transport of L-[^{14}C]threonine were studied with cellophane-grown cultures which had been transferred to Petri dishes containing 10 ml liquid medium and 4 mm glass beads (80 per plate) as a support for the cellophane. Labelled threonine (specific activity 10 mCi mol $^{-1}$) was obtained from The Radiochemical Centre (Amersham, Buckinghamshire). The final concentration of the labelled L-threonine in the growth medium was 0.1 $\mu\text{Ci ml}^{-1}$. After incubation, the mycelium was collected from the cellophane, washed with 0.1 M-phosphate buffer pH 7.0 until no radioactivity could be detected in the buffer, dried at 80 °C for 24 h, weighed and the radioactivity measured in a Packard Tri-Carb scintillation spectrometer model 3003.

Enzyme assays. The mycelium (approx. 3 g wet wt) was homogenized in 3 ml of suitable buffer, using an Ultra-Turrax (Janke & Kundel K.G., Staufen, West Germany), for 1 min at 4 °C, and the homogenate was centrifuged at 18000 g for 20 min at 4 °C. Unless otherwise stated, low molecular weight compounds were removed from the crude extract by overnight dialysis against distilled water at 4 °C. Protein content of the crude extract was determined with the Folin phenol reagent (Lowry *et al.*, 1951).

The following determinations were made on the crude extract, using the methods indicated. L-threonine dehydrogenase (Willetts & Turner, 1970); glycine decarboxylase (Willetts & Turner, 1971); 2-amino-3-oxobutyrate:CoA ligase, malate synthetase, L-serine hydroxymethyltransferase and CoA content (McGilvray & Morris, 1969); isocitrate lyase, glyoxylate dehydrogenase, oxalate and glyoxylate production (Maxwell & Bateman, 1968*b*); succinic dehydrogenase (Ulrich & Mathre, 1972); glucose 6-phosphate dehydrogenase (Caltrider & Gottlieb, 1963).

Glucan synthetase was determined by a modification of the method of Bartnicki-Garcia & Lippman (1972) for chitin synthetase. The fungus was homogenized in 0.1 M tris-HCl buffer pH 7.4 and the homogenate centrifuged at 27000 g for 30 min. One ml of the reaction mixture contained 0.14 nmol uridine diphospho[U- ^{14}C]glucose (specific activity 360 mCi mol $^{-1}$, The Radiochemical Centre), 0.5 μmol MgCl_2 , 0.5 μmol cellobiose, and 30 to 400 μg protein in 0.25 ml tris buffer. The reaction mixture was incubated for 1 h at 30 °C in a water bath, and then the reaction was stopped by immersion in boiling water for 1 min. Samples heated at zero time or lacking the enzyme extract were used as controls. After incubation, the reaction mixture was filtered through a Whatman no. 42 filter paper

and washed with 100 ml 0.4 M-NaOH/95 % (v/v) ethanol (1:7, v/v). The radioactivity remaining on the filter paper was measured and the values were taken as being proportional to enzyme activity; specific activity was expressed as c.p.m. (h incubation)⁻¹ (mg protein)⁻¹.

RESULTS

Effect of L-threonine and sodium oxalate on formation of sclerotia, branching pattern, glyoxylate and oxalate content

Sclerotium rolfsii type R was grown on cellophane-covered SM for 48 h and then transferred to Petri dishes containing the test substance(s). Strips of mycelium (0.5 cm wide) were collected at intervals from the colony edges, and examined for glyoxylate and oxalate.

When transferred to unsupplemented SM, the fungus covered the plate in 72 h; and the glyoxylate content at the colony margins started to increase on the 5th day, i.e. two days after the plate was covered with mycelium and sclerotial initials appeared (Fig. 1). In addition to the increase in glyoxylate content, a change in hyphal morphology (development of many branches) was seen 10 h after the fungus had been transferred to SM+L-threonine, the average distance (estimated from 25 replicates) between the newly-formed branches (internodes) being 46 μ m, compared with 375 μ m in the unsupplemented control. A sclerotial circle was formed 48 h after transferring the mycelium to L-threonine-supplemented medium and production of oxalic acid was significantly inhibited (0.01 mg/mg mycelium dry wt compared with 0.19 mg in the SM-grown fungus).

Cultures transferred to media containing sodium oxalate, or L-threonine+ sodium oxalate, showed a decrease in glyoxylate content at the colony margins (Fig. 1) with a concomitant decrease in branching (380 μ m between internodes) and a total inhibition of sclerotium formation.

L-[¹⁴C]threonine uptake

The uptake of labelled L-threonine by *S. rolfsii* was compared in media containing 0.1 M (on a nitrogen basis), NH₄Cl, NH₄NO₃ or NaNO₃ as a nitrogen source. The fungus was grown on cellophane-covered liquid SM for 48 h, transferred to threonine-supplemented medium and incubated for 2 h. Uptake (c.p.m./mg dry wt) was 1.97×10^6 , 2.3×10^6 and 4.6×10^6 , respectively, for the above nitrogen sources. Addition of 1.0 M-sodium oxalate to the medium did not affect uptake. No alteration in pH was observed after the termination of the experiment.

Effect on enzyme activity of adding supplements to growth media or reaction mixtures

L-Threonine dehydrogenase. Upon transfer of the fungus to threonine-supplemented SM, the activity of L-threonine dehydrogenase increased sharply and reached its peak after 30 h concomitantly with the initiation of the first circle of sclerotia (Fig. 2). Addition of sodium oxalate, either to the growth medium at a concentration of 1.5×10^{-2} M, or to the reaction mixture (10 μ mol) had no effect on the activity (Table 1). L-Cysteine and glycine, which inhibit sclerotium formation, strongly inhibited enzyme activity when added either to the medium or to the reaction mixture. Growth on ammonium nitrogen at relatively high concentration (10^{-1} M) yielded mycelium with a decreased enzyme activity. When added to the reaction mixture, however, 20 μ mol of ammonium nitrogen had no effect on the activity of L-threonine dehydrogenase.

Aminoacetone synthase and CoA content. Addition of L-threonine to the growth medium resulted in a 10-fold increase in the activity of aminoacetone synthase (2-amino-3-oxybutyrate:CoA ligase) in the cell-free extract, compared with the activity in a non-supplemented

Fig. 1

Fig. 2

Fig. 1. Effect of L-threonine and sodium oxalate on the glyoxylate content of *Sclerotium rolfsii*, type R. Mycelium was grown on SM for 48 h and then transferred to SM (○), SM + 10 mM L-threonine (●), SM + 15 mM-sodium oxalate (△), or SM + 10 mM L-threonine + 15 mM-sodium oxalate (▲).

Fig. 2. The activity of L-threonine dehydrogenase extracts prepared from *Sclerotium rolfsii*, type R. Colonies were transferred after 48 h growth on SM to SM (○), or to SM + 10 mM L-threonine (●). Activity is expressed as absorbance at 340 nm (mg protein)⁻¹ min⁻¹.

SM (Fig. 3). Glycine, added either to the growth medium or to the reaction mixture, caused a decrease in enzyme activity, whereas oxalate had no effect on either system (Table 1).

Except in glycine-grown fungus, which contained 0.036 µg CoA (mg protein)⁻¹, mycelium from other treatments contained 0.06 ± 0.005 µg CoA (mg protein)⁻¹.

Malate synthetase. The activity of this enzyme was greatly increased when *S. rolfsii* was transferred to threonine-supplemented medium, and decreased on transfer to SM containing ammonium or oxalate. Addition of ammonium (20 µmol) or oxalate (10 µmol) to the reaction mixture also inhibited enzyme activity, whereas threonine or glycine had no effect (Table 1). Six hours after the transfer of *S. rolfsii* to SM + threonine, there was a high level of enzyme activity, but this declined after 20 h of incubation (Fig. 4). After 85 h, there was a second significant increase in enzyme activity: this preceded the formation of a second sclerotial circle.

Isocitrate lyase. The activity of this enzyme in extracts obtained from threonine-grown *S. rolfsii* did not differ significantly from that of the control, but was higher in extracts of oxalate-grown fungus. On the other hand, the glyoxylate content of the mycelium of fungus grown on SM and SM + threonine was 10-fold higher than that of the oxalate-grown culture (Table 1).

Glyoxylate dehydrogenase. Transfer of *S. rolfsii* to SM + threonine resulted in a sharp decline in the activity of this enzyme in the mycelial extract (Table 1 and Fig. 5), as did the presence of ammonium chloride in the growth medium. The activity of this enzyme was also adversely affected by the addition of 10⁻⁵ M acetyl-CoA (Table 1).

Oxalate production. Transfer of *S. rolfsii* to SM + threonine resulted in a sharp decline in oxalate production (Table 1 and Fig. 6). However, the presence of ammonium chloride in the growth medium increased the production of oxalate as did the addition of oxalate to L-threonine supplemented medium.

Table 1. *Effect of L-threonine, L-cysteine, glycine, oxalate and ammonium chloride added to the growth medium or the reaction mixture on the activity of some enzymes involved in the glyoxylate cycle and threonine metabolism in Sclerotium rolfii*

Supplement	L-threonine dehydrogenase*	2-amino-3-oxo-butyrate: CoA ligase†	Malate synthetase†	Isocitrate lyase‡	Glyoxylate dehydrogenase*	Glycine decarboxylase¶	L-serine hydroxymethyl transferase¶	Glyoxylate§	Oxalate
None	0.009	0.028	0.09	0.42	0.270	4.24	1.41	10.5	0.120
Added to growth medium									
Sodium oxalate 15 mM	0.014	—	0.055	1.80	0.270	—	—	6.0	0.105
L-threonine 10 mM	0.110	0.217	1.05	0.39	0.026	11.96	13.00	75.0	0.010
L-cysteine 10 mM	0.002	—	—	—	—	—	—	—	—
Glycine 100 mM	0.010	0.23	—	—	—	—	—	—	—
NH ₄ Cl 100 mM	0.000	—	0.057	—	0.060	—	—	—	0.173
Sodium oxalate 15 mM } + L-threonine 10 mM }	0.100	0.193	0.096	1.98	0.253	—	—	4.8	0.086
L-cysteine 10 mM }	0.001	—	—	—	—	—	—	—	—
+ L-threonine 10 mM }	0.02	0.040	—	—	—	—	—	—	—
Glycine 100 mM }	0.047	—	0.047	—	0.006	—	—	—	0.008
NH ₄ Cl 100 mM }									
+ L-threonine 10 mM }									
Added to reaction mixture**									
L-threonine 10 µmol	—	—	1.02	—	0.168	—	—	—	—
L-cysteine 10 µmol	0.007	—	—	—	—	—	—	—	—
Sodium oxalate 10 µmol	0.106	0.190	0.00	0.00	—	—	—	—	—
NH ₄ Cl 20 µmol	0.110	—	0.175	—	—	—	—	—	—
Glycine 10 µmol	0.016	0.032	1.02	—	—	—	—	—	—
Acetyl-CoA 5 µmol	—	—	—	—	0.009	—	—	—	—
Acetyl-CoA †† 5 µmol	—	—	—	—	0.041	—	—	—	—

* Absorbance at 340 nm (mg protein)⁻¹ min⁻¹.

† Absorbance at 412 nm (mg protein)⁻¹ min⁻¹.

‡ µg glyoxylate (mg protein)⁻¹ min⁻¹.

§ µg glyoxylate (mg protein)⁻¹.

¶ mg oxalate (mg mycelium dry wt)⁻¹.

|| Absorbance at 355 nm (mg protein)⁻¹ min⁻¹.

** Mycelium from 10 mM L-threonine growth medium.

†† Mycelium from SM growth medium.

Fig. 3

Fig. 4

Fig. 3. The activity of 2-amino-3-oxobutylate:CoA ligase in extracts prepared from mycelium of *Sclerotium rolfsii*, type R. Colonies were transferred after 48 h growth on SM to SM (○), or to SM + 10 mM L-threonine (●). Activity is expressed as absorbance at 412 nm (mg protein)⁻¹ min⁻¹.

Fig. 4. Malate synthetase activity in extracts prepared from mycelium of *Sclerotium rolfsii*, type R, transferred after 48 h growth on SM to SM (○), or to SM + 10 mM L-threonine (●). Activity is expressed as absorbance at 412 nm (mg protein)⁻¹ min⁻¹.

Fig. 5

Fig. 6

Fig. 5. Glyoxylate dehydrogenase activity in *Sclerotium rolfsii*, type R, transferred after 48 h growth on SM to SM (○), or to SM + 10 mM L-threonine (●). Activity is expressed as absorbance at 340 nm (mg protein)⁻¹ min⁻¹.

Fig. 6. Production of oxalic acid by *Sclerotium rolfsii*, type R, transferred after 48 h growth on SM to SM (○), or to SM + 10 mM L-threonine (●).

Glycine decarboxylase and L-serine hydroxymethyltransferase. The activities of these enzymes, which were detected in mycelial extracts of *S. rolfsii*, were much higher when the fungus was grown on SM + threonine rather than SM alone (Table 1).

Glucan synthetase. Addition of L-threonine to SM significantly increased enzyme activity [60000 c.p.m. of labelled uridine diphosphoglucose incorporated into glucan compared with 10000 c.p.m. (h incubation)⁻¹ (mg protein)⁻¹ in SM-grown fungus] whereas sodium oxalate added to SM or SM + L-threonine caused a significant decrease in glucan synthetase activity [1600 ± 80 c.p.m. (h incubation)⁻¹ (mg protein)⁻¹]. No inhibition in enzyme activity was obtained when sodium oxalate (1, 10 or 100 μmol) was added to the reaction mixture.

Glucose 6-phosphate dehydrogenase and succinic dehydrogenase. No significant differences

in the specific activities of both glucose 6-phosphate dehydrogenase (50 to 70 units) and succinic dehydrogenase (170 to 200 units) were observed in the mycelium when the fungus was grown on either SM, SM + L-threonine, or SM + sodium oxalate media.

Metabolism of L-threonine

Sclerotium rolfsii was grown on cellophane-covered SM for 48 h, transferred to a medium containing labelled threonine ($0.1 \mu\text{Ci ml}^{-1}$), and placed in an anaerobic brewer's jar. Carbon dioxide produced during the first 2 h after transfer, was absorbed on to Whatman filter paper soaked in 4 M-KOH. The filter paper was removed, dried at room temperature and its radioactivity measured by scintillation counter. The rate of CO_2 production from L-threonine was influenced by the nitrogen source (0.1 M nitrogen basis) present in the medium, being 4.60×10^5 , 2.34×10^5 and 1.97×10^5 c.p.m. (g mycelium dry wt) $^{-1}$, for NaNO_3 , NH_4NO_3 and NH_4Cl , respectively.

In another experiment, the amounts of L-threonine, L-serine and glycine in the mycelial extract were examined by paper chromatography. Two hours after transfer to a medium supplemented with labelled L-threonine ($0.2 \mu\text{Ci ml}^{-1}$), an extract from about 2 mg wet wt of mycelia was spotted on to each paper. After development of the chromatograms with ninhydrin, the radioactivities of the spots were measured and found to be 66000 c.p.m., 2988 c.p.m. and 1753 c.p.m. for L-threonine, L-serine and glycine, respectively.

DISCUSSION

Mycelial extracts of threonine-grown *Sclerotium rolfsii* showed an increased activity of 2-amino-3-oxobutyrate:CoA ligase, glycine decarboxylase and L-serine hydroxymethyl-transferase, which operate the glycine-serine pathway. The favourable effect of L-threonine on the glyoxylate cycle could be explained by an increased production of acetyl-CoA and pyruvate, which both contribute to the production of malate, the central metabolite in this pathway. Indeed, the presence of malate synthetase in mycelial extracts of *S. rolfsii* and its increased activity in the threonine-grown fungus has also been demonstrated. The immediate output of $^{14}\text{CO}_2$ by *S. rolfsii* in the presence of L-threonine and the prominent accumulation of serine and glycine in the mycelium of the threonine-grown fungus indicate clearly that L-threonine is metabolized in *S. rolfsii* via the glycine-serine pathway.

The catabolic metabolism of L-threonine in *S. rolfsii* resembles threonine breakdown in *Penicillium* and *Fusarium* (Willetts, 1972*a, b*). As in *S. rolfsii*, most of the enzymes involved, i.e. L-threonine:NADH dehydrogenase, 2-amino-3-oxobutyrate:CoA ligase, isocitrate lyase and malate synthetase, were induced by L-threonine. However, *S. rolfsii* differs from *Penicillium* and *Fusarium* as well as from *Arthrobacter* (McGilvray & Morris, 1969) in not being able to utilize L-threonine as a sole source of carbon and nitrogen (unpublished results). Addition of sodium oxalate to the growth medium resulted in a decrease in glyoxylate content of the mycelium and inhibition of sclerotium formation in *S. rolfsii*. Sodium oxalate did not affect L-threonine uptake, but inhibited isocitrate lyase and malate synthetase activities. Oxalate also caused a decrease in branching at the colony margins and reduced glucan synthetase activity without directly inhibiting the enzymic reaction. Thus, L-threonine could favour sclerotium formation by derepression of the enzymes involved in branching and in cell-wall synthesis, brought about by increasing the supply of energy and metabolic precursors, (probably through its inhibitory effect on the production of oxalic acid) followed by increase in the level of acetyl-CoA, which inhibits glyoxylate dehydrogenase activity.

Induction of conidium formation in *Aspergillus* (Smith & Galbraith, 1971) and *Neurospora crassa* (Turian, 1973) was related to increased activity of the glyoxylate cycle.

In the present study, it was found that L-threonine increased glyoxylate content of the mycelium, branching at the colony margins and formation of sclerotia in *S. rolf sii*. The large quantities of oxalic acid normally produced from glyoxylate by the activity of glyoxylate dehydrogenase (Maxwell & Bateman, 1968*b*) were reduced by the addition of L-threonine to the growth medium.

Presumably, initiation, development and maturation of sclerotia in *S. rolf sii* require higher levels of energy and metabolites than the usual vegetative growth, these requirements being supplied by the glyoxylate cycle. Sclerotium formation is probably inhibited by glucose due to its effect as a catabolic repressor (Lehninger, 1970; Turian, 1973).

Although the glyoxylate cycle seems to play an important role in the morphogenetic processes of *S. rolf sii*, it is apparently not the only factor affecting formation of sclerotia. Similar levels of glyoxylate were observed in *S. rolf sii* types R and A, yet the efficiency of sclerotium formation is higher in type A (Chet & Henis, 1972).

No significant changes could be observed in the glucose 6-phosphate and succinic dehydrogenase activities upon transfer of *S. rolf sii* to any of the supplemented media, possibly indicating a lesser degree of involvement of the phosphogluconate pathway and tricarboxylic acid cycle in the formation of sclerotia in *S. rolf sii*.

Our results do not allow correlation of isocitrate lyase activity in the intact mycelium with its activity in the mycelial extracts. Sodium oxalate inhibited enzyme activity both *in vivo* and *in vitro* and reduced the glyoxylate level in the mycelium, yet the activity (measured *in vitro*) of isocitrate lyase in oxalate-grown *S. rolf sii* was much higher than in the fungus grown on a non-supplemented SM. Possibly, the lower intracellular levels of glyoxylate resulted in depression of isocitrate lyase synthesis. The glyoxylate content of the fungal mycelium is probably a better indicator of isocitrate lyase activity *in vivo* than enzymic activity in dialysed cell-free extracts.

It seems that formation of sclerotia in *S. rolf sii*, the catabolic metabolism of L-threonine, and the metabolic pathways involved, are controlled at the levels both of enzyme synthesis and activity.

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