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CONTROL OF METHIONINE SYNTHESIS BY LYSINE IN *LEMNA MINOR*

KWAN F. WONG* and EDWIN A. COSSINS
Department of Botany, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

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Key Word Index—*Lemma minor*; Lemnaceae; methionine synthesis; control by lysine; serine hydroxymethyltransferase; folate derivatives.

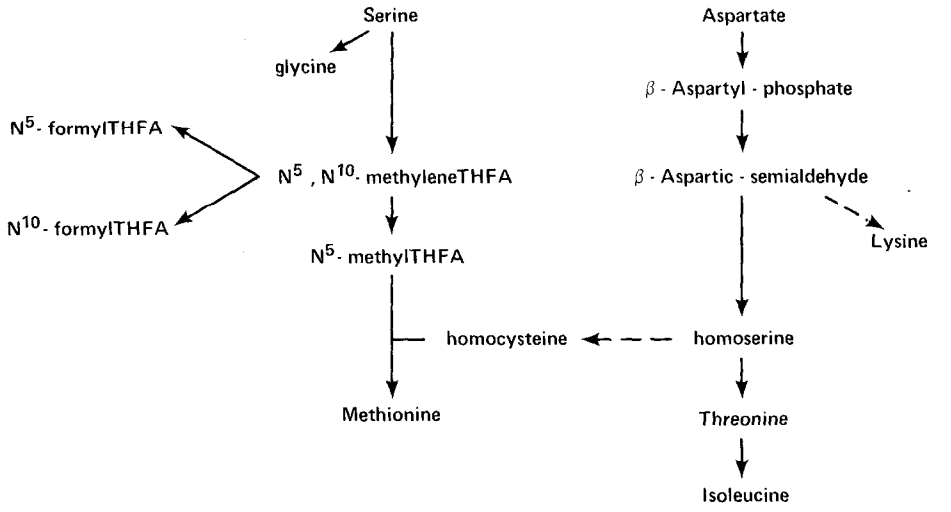
Abstract—When *Lemna minor* was cultured in the presence of 0.25 mM L-lysine, the concentration of free methionine and formyl and methyl tetrahydrofolate (THFA) were decreased. L-lysine, L-homoserine, L-threonine and L-methionine at concentrations up to 8 mM did not affect N^{10}-formyl THFA synthetase (E.C. 6.3.4.3) and N^5,N^{10}-methylene THFA reductase (E.C. 1.1.1.68). In contrast, serine hydroxymethyltransferase (E.C. 2.1.2.1) activity was inhibited by lysine. This inhibition gave a sigmoidal curve when plotted for a range of L-lysine or THFA concentrations. Exogenous lysine also reduced the incorporation of glycine [^{14}C] and serine [3-^{14}C] into free and protein methionine. Lysine, which is known to control synthesis of homocysteine in *L. minor*, may also regulate production of C-1 units for methionine synthesis by inhibition of serine hydroxymethyltransferase.

INTRODUCTION

Several metabolic pathways are known to be closely integrated so that alterations in one sequence result in changes in the products of related pathways [1,2]. This is referred to as interpathway regulation or metabolic interlock [3].

Regulation of this type occurs in microorganisms [4–6] and in higher plants [7,8]. The simplest examples involve interactions between individual branches of a multibranched pathway, mediated by allosteric inhibition or activation of key enzymes. In higher plants, the highly branched pathway of methionine–lysine–isoleucine biosynthesis (Scheme 1) could be regulated by this mechanism. In this respect, the branch leading to methionine derives a C_4 moiety from aspartate and a C-1 unit from serine. In *Neurospora crassa*, interaction between the pathways forming precursors of methionine centres on the cystathionine synthetase reaction [9]. In *Lemna minor* and wheat germ, homoserine formation is controlled by lysine and threonine through their inhibition of aspartokinase [10,11], and control of lysine and threonine synthesis may reduce the production of homocysteine and methionine. In this respect, Wong and Dennis [11] found that the inhibition of growth by lysine and threonine could be partially reversed by L-methionine. In *Marchantia polymorpha* [12] and *Mimulus cardinalis* [13,14], both lysine and

* Present address: Atlantic Regional Laboratory, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada.



Scheme 1. Interrelationships between pathways for lysine, isoleucine and methionine biosynthesis in higher plants.

Table 1. Effect of lysine on the levels of free methionine

Time after transfer (hr)	Free methionine—nmol/g fr. wt Experiment 1 Control	Experiment 1 Lysine	Experiment 2 Control	Experiment 2 Lysine
4	76	70	100	90
6	74	73	103	103
12	101	70	110	73
18	96	77	93	90
24	78	76	91	77

Plants were harvested after 10 days and transferred to complete medium with or without 0.25 mM lysine.

threonine reduced the incorporation of [^{14}C]aspartate into methionine. It is not known, however, if such conditions also affect the production of C-1 units from serine so that a balance between homocysteine production and methyl group biogenesis is achieved.

The present paper reports an effect of exogenous lysine on the pool sizes of methionine, formyl and methyl THFA which appears related to a partial inhibition of serine hydroxymethyltransferase by this amino acid.

RESULTS

After growth for 12 hr in medium supplemented with 0.25 mM L-lysine, the plants accumulated this amino acid to 3.3 μmol/g fr. wt compared to the 0.2 μmol/g fr. wt of the controls, and the concentrations of several neutral and basic amino acids increased. However, free methionine was decreased with greatest reduction after 12 hr (Table 1) and the levels of protein methionine, histidine and phenylalanine were 73, 78 and 80% respectively of the control plants. After 6 hr the concentrations of formyl and methyl THFA which existed as mono- and diglutamyl derivatives were lower in the lysine-treated plants (Table 2). After 12 hr the levels of these formyl derivatives were comparable to those of the control plants but the methyl folates remained lower. Other unidentified folate compounds collected later (150 fractions) in the elution from DEAE-cellulose were increased by lysine treatment (Table 2). These may be conjugated folates with at least three glutamyl residues [15]. In agreement with analyses of other higher plant species [15,16], the principal folates of *L. minor* occurred as polyglutamyl derivates. After growth for 6 and 12 hr in lysine supplemented medium the total polyglutamyl pool remained similar to that of the control plants.

Table 3. Effect of lysine on enzymes of C-1 metabolism

Enzyme	Control	Lysine
Serine hydroxymethyltransferase	0.18	0.11
N^5,N^{10}-methylene tetrahydrofolate reductase	0.02	0.02
N^{10}-formyl tetrahydrofolate synthetase	15.5	15.2

Enzyme activities were determined as described in Experimental. L-lysine was added to the standard reaction systems indicated to give the following final concentrations: serine hydroxymethyltransferase, 6.2 mM; N^5,N^{10}-methylene tetrahydrofolate reductase, 8 mM; and N^{10}-formyl tetrahydrofolate synthetase, 5.3 mM. Activities are expressed as nmol product formed in 10 min/mg protein and are the average of at least three separate assays.

As lysine affected the levels of formyl and methyl THFA, it was of interest to determine whether this amino acid would affect the activities of key enzymes of C-1 metabolism. Additions of lysine to desalted extracts of plants cultured in the absence of exogenous L-lysine inhibited serine hydroxymethyltransferase activity but did not affect the activities of N^{10}-formyl THFA synthetase or N^5,N^{10}-methylene THFA reductase. Additions of L-homoserine, L-threonine or L-methionine (up to 8 mM) had no effect on these three enzymes.

Attempts were made to purify serine hydroxymethyltransferase but this was difficult as 70% of the initial activity was lost within 5 hr of preparation at 2°. This loss could not be reduced by addition of 30% glycerol, 2% bovine serum albumin or by fractionating the protein with ammonium sulphate. Consequently we examined some properties of this enzyme immediately after desalting the crude extracts.

Although the enzyme did not show an absolute requirement for pyridoxal phosphate, activity was increased up to 3-fold by adding vitamin B_6 (0.2 mM). Isonicotinic acid hydrazide (4 mM) inhibited activity by 60% at pH 8. Enzyme activity was increased by raising the THFA concentration to 2.5 mM (Fig. 1). Above this concentration some inhibition was observed. The curve for activity as a function of THFA concentration was sigmoidal. The inhibitory effect of L-lysine was concen-

Table 2. Effect of lysine on individual folate derivatives

Derivative	Time after transfer 6 hr Control	6 hr % of total folate	6 hr Lysine	6 hr % of total folate	12 hr Control	12 hr % of total folate	12 hr Lysine	12 hr % of total folate
N^{10}-formyl THFA (mono- and diglu)	38.6	1.9	28.8	1.3	18.4	0.8	21.9	0.9
N^5-formyl THFA (monoglu)	50.0	2.5	24.0	1.0	48.0	2.2	46.0	2.1
N^5-methyl THFA (mono- and diglu)	447.0	22.3	417.0	17.7	525.0	24.0	468.0	21.0
Unidentified folates	120.0	6.0	210.0	8.9	122.0	5.5	176.0	7.9
Polyglutamyl folates	1340.0	67.2	1670.0	71.0	1474.0	67.3	1510.0	68.0
Total folates after γ-GCP treatment of extracts	2000.0		2350.0		2190.0		2220.0	

Plants were grown for 10 days and then transferred to complete medium with or without 0.25 mM L-lysine. Samples were taken for folate analyses as indicated. Data are expressed as ng folic acid equivalents/g fr. wt. γ-GCP = γ-glutamylcarboxypeptidase.

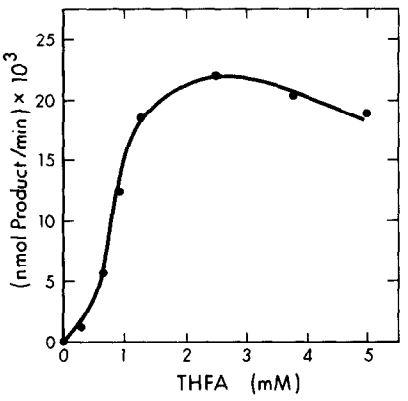


Figure 1. Effect of THFA concentration on serine hydroxymethyltransferase activity.

tration dependent and gave a sigmoidal curve (Fig. 2). Inhibition of serine hydroxymethyltransferase activity by L-lysine was not altered by raising the pyridoxal phosphate concentration nor did the addition of threonine and lysine together give a synergistic effect.

The sensitivity of *Lemna* serine hydroxymethyltransferase to lysine was greater than displayed by the corresponding enzyme from *Saccharomyces cerevisiae* (Table 4). In the presence of phosphate buffer, L-lysine (7.8 mM) inhibited the *Lemna* enzyme by 38% but the yeast enzyme was inhibited by only 13%. In Tes buffer, the *Lemna* enzyme was still inhibited by lysine whereas activity of the yeast enzyme was slightly stimulated. A further difference between these two enzymes was an apparent inhibition of the higher plant enzyme by phosphate buffer (Table 4).

The above data suggest that lysine reduces the flow of C-1 units for methionine synthesis. As glycine and serine are common precursors of methionine [23], ^{14}C feeding experiments were carried out to test the validity of this suggestion. Although both amino acids were rapidly taken up by control and lysine-treated plants, the carbons of glycine were more readily incorporated into methionine than C-3 of serine (Table 5). The high specific radioactivities of methionine following glycine [2-^{14}C] feeding indicated that some cleavage of this precursor occurred prior to methionine formation. Similarly, other analyses showed that the 2-carbon was preferentially incorporated into serine whereas the carboxyl carbon was extensively converted to carbon dioxide. Lysine-treated plants had less ability than the controls to form labelled methionine from either precursor (Table 5). In this respect lysine had most effect on the incorporation of C-1 of glycine into methionine. For serine [3-^{14}C], lysine had most effect in drastically reducing the labelling of protein methionine.

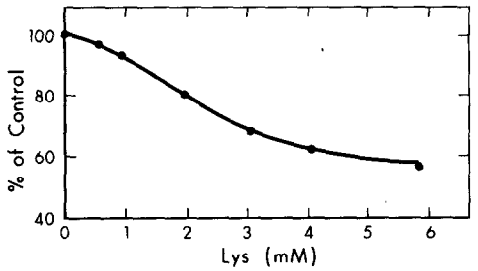


Figure 2. Effect of L-lysine concentration on the inhibition of serine hydroxymethyltransferase activity.

Table 4. Effects of lysine on yeast and *Lemna* serine hydroxymethyltransferase activity in phosphate and Tes buffers

Buffer (pH 8)	*Lemna* nmol/mg protein/10 min		Yeast nmol/mg protein/10 min	
	Control	Lysine	Control	Lysine
Phosphate buffer	0.110	0.077	0.122	0.106
Tes buffer	0.195	0.127	0.118	0.126

Enzyme activities were assayed as described in Experimental using 0.5 mg protein (yeast enzyme) and 0.4 mg protein (*Lemna* enzyme). L-lysine was added where indicated to give a final concentration of 7.8 mM.

DISCUSSION

The suggestion of Wong and Dennis [11] that culture of *L. minor* in lysine medium causes reduced synthesis of methionine, is supported by the analyses summarized in Table 1. It is perhaps not surprising that this treatment, which will affect synthesis of homocysteine precursors (Scheme 1), also affected production of C-1 units for methionine biosynthesis (Table 2). Reduced production of homocysteine could alone regulate methionine formation, but an overproduction of N^5-methyl THFA might occur without control of C-1 unit biosynthesis. As THFA derivatives are generally present in small concentrations. this situation could easily result in a serious imbalance within the C-1 pool. This is avoided in many species by control of methyl group biogenesis exerted at the levels of serine hydroxymethyltransferase [17–19] or N^5,N^{10}-methylene THFA reductase [20–22].

In *L. minor*, lysine can partially control methyl group biogenesis by affecting serine hydroxymethyltransferase activity (Fig. 2). As this reaction is generally regarded as the principal source of C-1 units [23] its partial inhibition should effectively reduce formation of both formyl and methyl THFA. Both types of derivatives declined in concentration when *Lemna* was grown in lysine medium for 6 hr (Table 2). Unfortunately, the un-

Table 5. The effect of lysine on incorporation of serine-[^{14}C] and glycine-[^{14}C] into free and protein methionine

Compound pulse fed for 30 min	^{14}C incorporated (cpm × 10^{-3})		Specific radioactivity (cpm × 10^{-3}/μmol)	
	Free	Protein	Free	Protein
Serine-[3-^{14}C]				
Control plants	1.54	8.86	38.4	4.7
Lysine-treated	1.20	1.80	30.7	0.9
Glycine-[1-^{14}C]				
Control plants	13.70	*n.d.*	392	—
Lysine-treated	5.69	*n.d.*	216	—
Glycine-[2-^{14}C]				
Control plants	25.90	7.79	863	1.86
Lysine-treated	15.40	4.18	700	1.00

n.d.—not detected.

stable nature of N^5,N^{10}-methylene THFA prevented its measurement. Lysine did not inhibit other key enzymes of C-1 metabolism so the changes observed in the folate pool probably reflect a decreased production of N^5,N^{10}-methylene THFA from serine. This was supported by the serine-[3-^{14}C] feeding experiments (Table 5) which showed that the lysine treatment reduced formation of labelled methionine. Lysine may also affect the formation of methylene THFA from glycine as reduced incorporations of glycine-[2-^{14}C] into methionine were also apparent in lysine-treated plants. This latter effect could, however, be indirect as we have no information on the sensitivity of glycine decarboxylase to L-lysine. In light of previous studies [10,11] the effect of lysine on incorporation of glycine-[1-^{14}C] into methionine may be partly related to synthesis via aspartate and homocysteine.

The sigmoidal responses of serine hydroxymethyltransferase to increasing concentrations of THFA and lysine (Figs. 1 and 2) suggest that it is a regulatory enzyme. This conclusion is also consistent with the acknowledged role of this enzyme which occupies a central position in C-1 metabolism. However, it should be emphasized that the enzyme was only partially inhibited by lysine even at concentrations as high as 6 mM. Thus, some production of C-1 units might occur even though lysine was reducing homocysteine production through its regulation of aspartokinase activity. In this respect *Lemna* may contain isoenzymes of serine hydroxymethyltransferase so that C-1 units, arising from serine, could be effectively channelled into different pathways of folate metabolism. In this regard, N^5,N^{10}-methylene THFA, required for methionine synthesis, may be formed by an isoenzyme whose activity in *Lemna* is regulated by excess lysine. There is, in fact, growing evidence that the pathways of C-1 metabolism in plants are highly compartmented [16,24]. It would be of interest to determine whether the reactions of homocysteine synthesis and methyl group biogenesis which occur in pea mitochondria [25] also occupy a common compartment in *Lemna*.

In yeast, lysine is synthesized by a pathway involving α-aminoadipate [26] and accordingly does not regulate aspartokinase activity [27]. Thus the pathways of lysine and methionine biosynthesis are not so closely related as in higher plants. Therefore, in yeast, a control of methionine biosynthesis by lysine would not be anticipated. The finding (Table 4) that yeast serine hydroxymethyltransferase was not inhibited by lysine supports this expectation. Where close relationships exist, however, as in *Lemna*, partial inhibition of this key enzyme by lysine may be a cellular device to integrate related metabolic pathways.

EXPERIMENTAL

Chemicals. Serine-[3-^{14}C] (56.5 μCi/μmol), glycine-[1-^{14}C] (57 μCi/μmol), glycine-[2-^{14}C] (57 μCi/μmol) and THFA-N^5-[*methyl*-^{14}C] (60 μCi/μmol) were purchased from the Radiochemical Centre, Amersham, England. All reagents were of the highest quality commercially available and were used without further purification. Solutions of THFA were freshly prepared in 1 mM 2-mercaptoethanol.

Plant material and enzyme preparation. Lemna minor L. was maintained in sterile culture as previously described [11]. To study the effects of L-lysine, plants which had been grown for 10 days were harvested, washed in sterile H_2O, and transferred to fresh medium supplemented (0.25 mM) with lysine. Cultures were maintained at 27° and received continuous light with an intensity of 1000 lx. In enzyme studies, tissue samples were ground in 2 vol. of 0.1 M KPi buffer (pH 7) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was passed through 8 layers of cheesecloth and centrifuged at 10000 *g* for 15 min. A (5-ml) aliquot of the supernatant was applied to a column of Sephadex G-25 (2 × 30 cm) and eluted with 0.1 M KPi buffer (pH 8) containing 1 mM EDTA. All procedures were carried out in triplicate at 4°. Triplicate enzyme assays were immediately carried out on the desalted extracts. Preliminary assays were carried out in all cases to establish conditions for optimal activity.

Serine hydroxymethyltransferase (E.C. 2.1.2.1) activity was assayed essentially by the method of ref. [28]. The reaction mixture, containing 67 mM KPi buffer (pH 8), 0.21 mM pyridoxal-5-phosphate, 2 mM THFA and plant extract (0.5 mg protein) was incubated at 27° for 5 min and 1.3 μM of serine-[3-^{14}C] (0.05 μCi of ^{14}C) was then added. After incubation for a further 10 min, the reaction was terminated by addition of 0.3 ml of 1 M NaOAc (pH 4.5) followed by 0.2 ml of 0.1 M HCHO and 0.3 ml of 0.4 M dimedone. The mixtures were heated for 5 min at 100°, cooled at 0° and the dimedone adduct extracted with 3 ml of toluene. Aliquots of the toluene extract were assayed for radioactivity [29]. Control systems lacking THFA were included in each assay.

N^5,N^{10}-*methylene THFA reductase* (E.C. 1.1.1.68) was assayed by the method of ref [30]. Each reaction mixture contained 80 mM KPi buffer (pH 6.5), 20 μM FAD, 20 μM menadione, 20 mM HCHO, 6.4 μM N^5-THFA-[methyl-^{14}C] (0.1 μCi of ^{14}C) and plant extract (0.2 mg protein). The mixtures were incubated for 10 min at 27°.

N^{10}-*formyl THFA synthetase* (E.C. 6.3.4.3) was assayed according to ref [31]. Each reaction contained 53.3 mM triethanolamine (pH 8), 0.2 M Tris–formate, 2.7 mM ATP, 1.5 mM THFA, 7.9 mM $MgCl_2$, 0.27 M KCl, 13.7 mM mercaptoethanol, and 0.5 mg extract protein. The reaction was terminated after incubation at 27° for 10 min. The extinction coefficient for N^5,N^{10}-methenyl THFA at 350 nm was taken as 22×10^6 cm^2/mol [31].

Extraction of amino acids and protein. After killing the plants in boiling 80% EtOH, free amino acids were extracted by washing in 50% EtOH and H_2O followed by fractionation on Dowex resins [32]. Amino acid pool sizes were determined [33] with an amino acid analyzer. Protein was extracted by the method of Trewavas [34] and hydrolyzed [35] for subsequent analysis of protein amino acids. Soluble protein was measured colorimetrically by biuret and Folin–Ciocalteu methods [35].

Extraction and assay of folate derivatives. Samples of harvested plants (1 g fr. wt) were killed by boiling in 10 ml of 2% (w/v) K ascorbate (pH 6) for 10 min. After rapid cooling at 0°, the plants were homogenized using a hand grinder. Following centrifugation (10000 *g* for 10 min) the supernatant was retained for folate analysis. Polyglutamyl folates were hydrolyzed by treatment with a carboxypeptidase isolated from pea cotyldeons [36]. Individual derivatives were separated by DEAE-cellulose column chromatography followed by differential microbiological assay [36].

Preparation of yeast serine hydroxymethyltransferase. Saccharomyces cerevisiae (ATCC 9763) was maintained on wort slants and transferred to a defined minimal liquid culture medium [37] for aerobic growth at 30°. Cells were harvested after 24 hr, washed and suspended in 0.1 M KPi buffer (pH 7) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. Cell-free extracts were prepared by sonication and subsequent centrifugation. After desalting by passage through columns of Sephadex G-25, serine hydroxymethyltransferase activity was measured as described above.

^{14}*C feeding experiments.* Plants were grown and treated with L-lysine as described above. After harvesting, 1 g fr. wt samples were placed in 100 ml control flasks fitted with a centre well and containing 8 ml H_2O. 2 μCi of glycine-[2-^{14}C] (57 μCi/μmol), glycine-[1-^{14}C] (57 μCi/μmol) and serine-[3-^{14}C]

(56.5 μCi/μmol) respectively were then added and incubation at 27° under room light continued for a further 30 min with gentle shaking. Liberated CO_2 was absorbed on a filter paper wick moistened with 20% KOH. At the end of the feeding period, the plants were washed at 2° on a funnel with a 1 mM soln of the corresponding amino acid, rinsed in H_2O and killed by boiling in 80% (v/v) EtOH for 5 min. After thorough grinding in a glass homogenizer and centrifugation, the residue was washed successively with 50% (v/v) EtOH and H_2O. The combined supernatants were fractionated using ion-exchange resins as previously described [38]. Free and protein amino acids were isolated with an amino acid analyzer [21].

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