COEXISTENCE OF TWO PATHWAYS OF SPERMIDINE BIOSYNTHESIS IN *LATHYRUS SATIVUS* SEEDLINGS

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1. Introduction

In most organisms, *S*-adenosyl-L-methionine decarboxylase (SAMDC EC 4.1.1.50) plays a pivotal role in polyamine biogenesis by contributing *S*-methyladenosylhomocysteamine which in turn donates its propylamine moiety in a stepwise manner to putrescine and spermidine to yield spermidine and spermine, respectively [1–3]. Two distinct and separable enzymes, namely spermidine synthase (EC 2.5.1.16) and spermine synthase, catalyze these transfer reactions [2]. A distinguishing feature of the SAMDC from higher animals and yeast is its activation by putrescine and to a lesser extent by spermidine, presumably to ensure adequate *S*-methyladenosylhomocysteamine for enhanced spermidine production [4,5] and hence elevated spermidine/spermine ratios during growth and development; concurrent inhibition of spermine synthase by the diamine may be a contributory factor in this regulatory phenomenon [4]. While the lack of putrescine activation of the prokaryotic and some lower eukaryotic SAMDCs can be understood in view of near absence of spermine and its synthase in these species [4,6], the presence and augmented production of both the polyamines in the developing higher plants [7,8] is not easily explained in the absence of the above regulatory mechanism.

We reported the purification and some properties of the biosynthetic SAMDC from *Lathyrus sativus* seedlings and showed that it was of prokaryotic type in being Mg^{2+}-dependent, but putrescine-insensitive [9]. Both the specific and total enzyme activities were, however, too feeble to account for large increases in concentrations of the polyamines and in the elevated spermidine/spermine ratios during the seedling growth [8–10], raising the possibility of additional modes of spermidine biogenesis. Of relevance in this context was the demonstration [11] that in some prokaryotes lacking detectable SAMDC activity, a novel route of spermidine synthesis prevails. According to the proposed scheme, aspartic β-semialdehyde forms a Schiff base with putrescine to be enzymatically reduced by an NADPH-dependent step to yield 'carboxyspermidine' which in turn undergoes a pyridoxal phosphate-dependent enzymatic decarboxylation to give rise to spermidine. Here we provide evidence for the coexistence of both the classical SAMDC pathway and the new route of spermidine biosynthesis in *Lathyrus sativus* seedlings, and show that the latter biosynthetic sequence is primarily restricted to spermidine synthesis.

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2. Materials and methods

2.1. *Chemicals*

[1,4-^{14}C]Putrescine . 2 HCl (55 mCi/mmol), [U-^{14}C]aspartic acid (227 mCi/mmol) and [G-^{3}H]methionine (290 mCi/mmol) were from the Radiochemical Centre, Amersham. All other chemicals were of analytical grade. Aspartic β-semialdehyde was synthesized by ozonolysis of DL-allyl glycine and was purified prior to use [12].

2.2. *Synthesis and characterization of carboxyspermidine*

Carboxyspermidine was synthesized by incubating aspartic β-semialdehyde (100 μmol) with putrescine (100 μmol) at pH 7.0 for 1 h at 22°C and the resul-

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tant Schiff base was reduced with an excess of $NaBH_4$. After destroying the unreacted $NaBH_4$ by exposure to acid pH, the reaction mixture was adjusted to pH 6.0 and applied on a phosphocellulose column (3.5 × 20 cm) [13] for removal of aspartic β-semialdehyde by exclusion and of putrescine with 0.05 M HCl prior to recovery of carboxyspermidine by elution with 0.2 M HCl. Carboxyspermidine labelled in the tetramethylene moiety was similarly synthesized using labelled putrescine. The synthetic compound migrated as a single ninhydrin-positive band on electrophoresis on paper (0.03 M sodium citrate buffer (pH 4.6)) [14], well separated from both spermidine and putrescine, with a slower mobility than the former. Its DNP-derivative had an A_{350}/A_{390} of 1.45 which was similar to that of spermidine [15] but, unlike spermidine, was not extractable into butanol under highly alkaline conditions (pH 12–13).

2.3. *Other methods*

The source, germination conditions of *L. sativus* seeds and the initial steps of enzyme extraction and purification from the seedlings were the same as in [16]. Infiltration of the labelled amino acids into 3-day-old *L. sativus* shoots was carried out under partial vacuum [17] and the shoots were allowed to metabolize them prior to the isolation of the labelled amine fraction using a Dowex-50 (H^+) column (3.8 × 11 cm) [18]. The labelled amines were resolved by chromatography [19] and the associated radioactivity quantified as in [9]. Protein was determined by the method in [20].

2.4. *Enzyme assays*

The production of carboxyspermidine was assayed in a reaction mixture (1.0 ml) containing potassium phosphate buffer (pH 7.5, 50 μmol), L-threonine (5 μmol), dithiothreitol (3 μmol), putrescine . 2 HCl (10 μmol) or [^{14}C]putrescine (0.25 μmol 0.075 μCi), aspartic β-semialdehyde (10 μmol) NADPH (0.5 μmol) and enzyme protein (0.1–1.5 mg), incubated for 1 h at 37°C. After termination of the reaction with 0.1 ml 20% $HClO_4$, the supernatant was adjusted to pH 6.0, carboxyspermidine was fractionated on phosphocellulose, purified by paper electrophoresis as described earlier and quantified by ninhydrin reaction [21] or measuring the radioactivity.

The decarboxylation of carboxyspermidine was assayed in a reaction mixture (1.0 ml) containing Tris–HCl (pH 8.4, 30 μmol), dithiothreitol (5 μmol), pyridoxal phosphate (20 nmol), $MgCl_2$ (5 μmol) and 0.5 μmol carboxyspermidine (5000 cpm) and incubated at 37°C for 2 h. The labelled spermidine formed was extracted with butanol under alkaline conditions after stopping the reaction. The acidified butanol fraction was evaporated in vacuuo and the radioactivity was counted by liquid scintillation spectrometry [9].

3. Results and discussion

3.1. *Infiltration experiments*

Since aspartic acid is the ultimate precursor of the propylamine moiety of spermidine in certain Gram positive and photosynthetic bacteria [11], infiltration of *L. sativus* embryo axis with the ^{14}C-labelled amino acid and its subsequent in vivo conversion to aliphatic amines were examined. Parallel feeding experiments with labelled methionine were conducted to confirm the coexistence of the classical pathway of polyamine biogenesis and to get a comparative estimate of the relative distribution of radioactivity in the polyamines. The pattern of incorporation of radioactivity into putrescine, spermidine and spermine under these conditions is shown in table 1. The finding that significant amounts of radioactivity from these amino acids were associated with the purified amines is indicative of the operation of both pathways. With [^{14}C]aspartic acid, nearly 72% of total radioactivity in the amine fraction was localized in spermidine while that encountered in spermine was ~10-times less. In terms of specific activity spermidine was 4.5-times superior to spermine which however was 2.2.-times less in concentration than spermidine [10]. On feeding [^{3}H]methionine, % total radioactivity incorporated into spermidine was still the highest, being comparable to that obtained with [^{14}C]aspartic acid. But the magnitude of difference in total radioactivity in the 2 polyamines was considerably less in this case, with the label in spermidine being only 2.5-times more than that in spermine. This together with the close similarity in specific activities of the 2 polyamines connected by a precursor–product relationship suggests that methionine is more efficient precursor of spermine and that the utilization of aspartic acid as the propylamine donor is mostly restricted to spermidine synthesis. The appearance of significant amount of radioactivity in putrescine with

Table 1
Incorporation of radioactivity from [U-^{14}C]aspartic acid and [G-^{3}H]methionine into polyamines after infiltration into *L. sativus* embryo axis

Amine	Infiltration of					
	[U-^{14}C]aspartic acid			[G-^{3}H]methionine		
	Total counts recovered	% incorporation	Spec. act. (cpm/μmol)	Total counts recovered	% incorporation	Spec. act. (cpm/μmol)
Putrescine	4500	20	3300	–	–	–
Spermidine	16 560	72	82 800	29 760	70	102 600
Spermine	1800	7	18 000	12 600	30	93 800

Batches of 15 embryo axes of *L. sativus* were supplied with 10 μCi [U-^{14}C]aspartic acid or [G-^{3}H]methionine and the labelled amines isolated after 20 h and quantified as in section 2

the labelled aspartic acid, but not with methionine, is not easily explained at present and may be related to channeling of the label from aspartic acid through metabolic conversions distantly related to the diamine biosynthesis.

Further evidence for the operation of 'aspartic acid' pathway of spermidine synthesis stems from enzymatic studies. Incubation of the dialyzed crude extracts with [^{14}C]putrescine, NADPH, dithiothreitol, $MgCl_2$ and pyridoxal phosphate, and either aspartic β-semialdehyde or homoserine, led to the formation of significant amounts of labelled spermidine.

3.2. *Characterization of carboxyspermidine as an intermediate enzymic product*

The recognition that exogenous pyridoxal phosphate in the reaction mixture was essential for labelled spermidine production led to the isolation and characterization of a labelled small molecular weight intermediate product as carboxyspermidine which unlike spermidine could not be extracted into butanol under alkaline conditions [11]. In attempts to purify this radioactive derivative, the butanol-insoluble fraction was applied to a Dowex-50 (H^+) column (2.8 × 10 cm) at pH 5.0 along with 500 nmol synthetic carboxyspermidine. After washing with I M HCl to remove salts, the radioactive product coeluted with carboxyspermidine (used as the carrier) on elution with 6 N HCl. The eluate after concentration and electrophoresis on paper showed that most of the radioactivity comigrated with a sharp ninhydrin-positive band corresponding to the position of authentic carboxyspermidine. It could also be demonstrated that substitution of unlabelled putrescine for the radioactive diamine resulted in detectable amount of carboxyspermidine. The quantity of carboxyspermidine produced was proportional to the time of incubation (10–60 min) and the enzyme protein (0.3–1.5 mg).

3.3. *Purification of carboxyspermidine synthase (putrescine–aspartic β-semialdehyde Schiff base reductase)*

Since an NADPH-dependent step is involved in the enzymatic synthesis of carboxyspermidine, partial purification of the Schiff base reductase was attempted employing blue Sepharose as the affinity matrix [22].

Table 2 summarizes the small scale protocol employed for the purification, with a resultant increase in specific activity by 45-fold and 52% recovery. The elution of the enzyme activity from the blue Sepharose was specific to $NADP^+$ since 5-fold higher [NAD^+] could not achieve elution of the enzyme from the affinity matrix.

The enzyme had a pH optimum of 7.5, was stable at 4°C for 7 days and had absolute requirement of NADPH (table 3). Addition of 5 μmol L-threonine stimulated the activity by 150%, presumably due to interference with the draining of aspartate β-semialdehyde by conversion to L-homoserine in the crude extracts. Cadaverine and 1,3-diaminopropane, at concentrations equivalent to that of putrescine, seem to act as substrates, but with ~30% efficiency. Spermidine (1 mM) caused 30% inhibition in enzyme activity which also appears to be SH-dependent since *N*-ethylmaleimide (NEM) at high concentration (>3 mM) drastically interfered with the enzyme.

Table 2
Partial purification of carboxyspermidine synthase from *L. sativus* seedlings

Step	Protein (mg)	Total activity (units)	Spec. act. (units/mg protein)	Purification (-fold)	Yield (%)
1. Crude extract	1280	30.0	0.023	1.0	100
2. $MnSO_4$ supernatant	1080	28.1	0.026	1.1	93
3. Blue Sepharose affinity-$NADP^+$ specific elution	15	15.7	1.040	45.0	52

The clarified $MnSO_4$ supernatant fraction, after removal of nucleoproteins [16], was applied on to a blue Sepharose column (3.5 × 20 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 containing 2 mM β-mercaptoethanol. Following extensive washing, elution of the enzyme with 5 mM NAD^+ and 1 mM $NADP^+$ in the equilibration buffer was carried out successively. NAD^+ elution did not dislodge the enzyme. One unit of enzyme activity is defined as 1 μmol carboxyspermidine formed . mg $protein^{-1}$. h^{-1}

3.4. *Carboxyspermidine decarboxylase*

The presence of this enzyme activity in the crude extracts could be demonstrated by quantitating the spermidine formed following incubation with pyridoxal phosphate and carboxyspermidine. However, the activity was too feeble and this was further compounded by the relatively low specific radioactivity of synthetic carboxyspermidine (2000 cpm/μmol). This activity was SH-dependent since NEM (1 mM) decreased the enzyme activity by 40%. It has inhibited by NSD-1055 (4-bromo-3-hydroxybenzoyloxyamine dihydrogenphosphate), an inhibitor of pyridoxal phosphate requiring enzymes [23], by 60%. In contrast, equivalent concentration of MGBG (methyl guanylbis (guanylhydrazone)

Table 3
Requirements for the carboxyspermidine synthase reaction

Component omitted	cpm in carboxyspermidine formed
None	6000
NADPH	100
Aspartic β-semialdehyde	50
Boiled enzyme preparation used instead of active enzyme	120

The complete reaction mixture contained potassium phosphate buffer 50 μmol (pH 7.5), dithiothreitol 2 μmol, aspartic β-semialdehyde 5 μmol [^{14}C]putrescine 0.5 μmol; 50 000 cpm, NADPH 0.5 μmol and 100 μg of the partially pure enzyme protein. After the termination of reaction, 0.5 μmol synthetic carboxyspermidine was added as the carrier and the product isolated, purified and radioactivity quantified as in the text

which has relatively specific and pronounced inhibitory action on SAMDC's from several biological systems [1,3] including *L. sativus* [9], exhibited only marginal (<10%) inhibition of the carboxyspermidine decarboxylase activity. Spermidine (1 mM) caused a 70% inhibition of this enzyme.

From the foregoing, evidence for the operation in *L. sativus* of both pathways of polyamine biogenesis is unequivocal. One of these utilizes methionine through the classical pathway involving SAMDC [1–3], whereas in the other, aspartic acid functions as the ultimate precursor of the propylamino group of spermidine through carboxyspermidine as the intermediate [11]. While the functioning of the latter route as the sole pathway for spermidine synthesis, in complete absence of the former, in certain microorganisms [11] is understandable, the evolutionary implications of the coexistence of both pathways in a higher plant are not readily apparent. It is conceivable, that the plants with a prokaryotic-type SAMDC [6,9] lack the versatile built-in mechanism of putrescine activation of SAMDC to drive their biosynthetic machinery for polyamine synthesis to meet physiological demand and hence have retained this additional mechanism of spermidine synthesis during evolution. Furthermore, it is well documented that in several organisms, the ready availability of *S*-adenosylmethionine for a plethora of biosynthetic reactions involving methylation of vital biomolecules, is limited by the low concentration of methionine in vivo [3,24]. Since the leguminous plants in general have limiting concentration of this amino acid in their tissues [25], an alternate mechanism of spermidine

synthesis employing the relatively abundant aspartic acid might bestow on them adequate capacity of spermidine production. Apart from being a physiological necessity, the question of preferential utilization of this new pathway for spermidine synthesis vis-a-vis spermine may be related to the specificities of the enzymatic recognition of appropriate type of Schiff bases formed prior to reduction and subsequent decarboxylation of the carboxy derivatives. A similar explanation may be valid for the absence of spermine in those organisms in which the aspartic acid pathway is the sole route of polyamine synthesis [6,11]. It is interesting that both modes of propylamine transfer are subjected to strict regulation by inhibition by end products [26–28]. However, what biochemical and physiological in vivo signals dictate the relative contribution of the two pathways of spermidine synthesis in *L. sativus*, needs further study.

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