Biosynthesis of 5-aminolevulinic acid via the Shemin pathway in a green sugar beet callus

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Abstract

5-Aminolevulinic acid synthase (ALAS) has been detected in a normal (auxin- and cytokinin-dependent) green sugar beet callus under light and under darkness. ALAS activity was lower when the callus was grown under light. The supply of precursors of the Shemin pathway (glycine and succinate) to dark-grown callus enhanced considerably the capacity of the 5-aminolevulinic acid (ALA) formation. Glutamate, γ-aminobutyrate or α-ketoglutarate also increased ALA accumulation. Such an accumulation was also obtained after inhibition of polyamine synthesis. The results show that glutamate or its derivatives might feed the Shemin pathway in conditions preventing glutamate to be used through the Beale pathway.

Additional key words: 5-aminolevulinic acid synthase, *Beta vulgaris*, heterotrophy.

Introduction

The common precursor for all tetrapyrroles is 5 aminolevulinic acid (ALA). It is now generally assumed that in higher plants and most green algae ALA is synthesized via the light-dependent C-5 pathway in the plastid, called Beale pathway (Reinbothe and Reinbothe 1996). In animal, yeast and fungi cells, and in some bacteria (including the purple sulfur bacteria) ALA is formed by the Shemin pathway, via the condensation of succinyl CoA and glycine by ALA synthase (Beale and Weinstein 1989). The phytoflagellate *Euglena gracilis* (Weinstein and Beale 1983) and the green alga *Scenedesmus obliquus* (Dreschsler-Thielmann *et al.* 1993) have both ALA-forming pathways. Plastid tetrapyrroles are formed from glutamate, while mitochondrial hemes are formed from glycine and succinyl CoA. It is difficult to extrapolate from

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Abbreviations: ALA - 5-aminolevulinic acid; ALAS - 5-aminolevulinic acid synthase; DFMO - difluoromethylornithine; DFMA - difluoromethylarginine, GABA - γ-aminobutyrate.
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these results to the situation occurring in dark-grown higher plant tissues. It has been previously shown that the transfer of light-cultured green sugar beet callus to darkness does not abolish completely its growth. The decreased CO_2 fixation was compensated by an increased dependency on externally supplied sucrose (Kevers *et al.* 1995).

Our first aim was to find out whether the Shemin pathway can provide ALA under darkness instead of the C-5 light-dependent pathway, which is unable to work in these heterotrophic conditions. Our second aim was to determine whether glutamate (the natural precursor of C-5 pathway) or by-products might enter the metabolic flow leading to ALA synthesis by the Shemin pathway under darkness.

Materials and methods

Experimental conditions for obtaining green normal callus of sugar beet (*Beta vulgaris* L. ssp. *altissima*), and for maintaining this tissue in solid stock culture under light (16-h photoperiod, irradiance of 17 W m^{-2} provided by *Sylvania Grolux* fluorescent tubes, temperature of 25 °C) or under darkness have been reported elsewhere (Kevers *et al.* 1981, 1995). Such calli were subcultured (transfer of 200 to 500 mg of callus to 70 cm^3 plastic dishes) every 2 weeks on their solid medium [basal medium supplemented with 0.1 g dm^{-3} 2,4 dichlorophenoxyacetic acid (2,4 D) and 0.1 g dm^{-3} benzylaminopurine (BAP)], in the absence or in the presence of precursors of the Shemin pathway or of inhibitors of the polyamine synthesis.

The capacity of ALA synthesis was determined by blocking the 5-aminolevulinate dehydratase (ALAD). The callus was treated 48 h before ALA measurement with 5 mM levulinic acid which is a competitive inhibitor of ALAD. The extraction and determination of ALA were monitored according to a modified method of Beale *et al.* (1975).

Callus fresh mass (1 g) was ground in perchloric acid (0.5 M) and centrifuged at 10 000 *g* during 15 min at 0 °C. The supernatant was adjusted to pH 2 by KOH and incubated 12 h at 4 °C. The excess of KCl was eliminated by centrifugation. The supernatant was then deposited on a resin (*Biorad 50W-W8*; 200 - 400 mesh) in Na^+ form washed by sodium nitrate buffer 0.2 M (pH 3.07). The ALA was eluted by a citrate-Na^+ buffer 0.2 M (pH 5.25). 0.04 cm^3 of acetylketone were added to the eluted solution and incubated for 10 min at 100 °C. The ALA accumulation was measured by the addition of the Ehrlich reagent [acetic acid (42 cm^3), perchloric acid (10 cm^3), *p*-dimethylaminobenzaldehyde (1 g) and mercuric chloride (0.175 g)]. After 10 min incubation the absorbance of the colored complex was measured at 553 nm at 25 °C. The results (means of three separate assays) are expressed in [nmol(ALA) g^{-1}(f.m.)].

For 5-aminolevulinic acid synthase (ALAS) assay, callus (1 g) was ground in 2 cm^3 of 100 mM Tris-HCl buffer (pH 7.8) containing 0.35 M NaCl. After centrifugation (20 000 *g*, 20 min, 5 °C) the supernatant was desalted on *Sephadex G-25* and assayed for ALAS activity according to Ramaswamy and Nair (1973). Proteins in the enzyme preparations were estimated using the procedure of Bradford (1976). Each analysis was repeated at least in triplicate.

Results and discussion

The activity of 5-aminolevulinic acid synthase (ALAS) was higher in dark-grown sugar beet callus than in light-grown callus (Table 1). The dark enhancement of ALAS activity has also been described in *Euglena* (Foley *et al.* 1982). The Shemin

Table 1. The activity of 5-aminolevulinic acid synthase (ALAS) in sugar beet callus grown for 7 or 14 d under light or under darkness. Values are means of three separate assays ± SE.

Culture duration [d]	ALAS [μmol(ALA) mg^{-1}(protein) s^{-1}]	
	light	darkness
7	2.50 ± 0.10	3.66 ± 0.11
14	3.24 ± 0.15	4.53 ± 0.21

pathway might compensate the inability of the C-5 pathway to function in darkness. The capacity of ALA synthesis has been determined by measuring ALA accumulation in levulinate-treated calli (Table 2) grown under darkness. In these conditions, the supply of direct precursors (glycine and succinate) of the Shemin pathway increased the ALA content. This result was in agreement with the involvement of ALAS in ALA synthesis. One can wonder whether glutamate which is the precursor of the Beale pathway might be an indirect precursor of the Shemin

Table 2. Effect of precursors of 5-aminolevulinic acid (ALA) and inhibitors of polyamine biosynthesis pathway on ALA content of levulinate-treated (5 mM) sugar beet callus grown under darkness. Means of three separate assays ± SE.

	ALA content [nmol g^{-1}(f.m.)]
Control	11.7 ± 0.8
10 mM glycine + 10 mM succinate	22.3 ± 1.1
10 mM glutamate	17.2 ± 1.3
10 mM α-ketoglutarate	18.6 ± 1.9
10 mM γ-aminobutyrate	29.1 ± 2.7
2 mM difluoromethylornithine	16.7 ± 1.5
2 mM difluoromethylarginine	20.9 ± 2.1

pathway in heterotrophic conditions. Indeed, glutamate can be transformed into succinate (Fig. 1) either by its metabolization by the Krebs cycle or by the γ-amino-butyrate (GABA) shunt (Bisbis *et al.* 1997). Our results showed that not only glutamate itself but also GABA or α-ketoglutarate increased the capacity of ALA synthesis. One way to increase the glutamate pool in heterotrophic conditions was to block the polyamine formation without impairing notably the growth of callus. The supply of difluoromethylornithine (DFMO) or difluoromethylarginine (DFMA) which are the respective inhibitors of ornithine decarboxylase or arginine

decarboxylase (Burtin *et al.* 1989) provoked an important increase of ALA synthesis. It thus might be assumed that glutamate not used for polyamine synthesis led to succinate formation via the Krebs cycle or the GABA shunt.

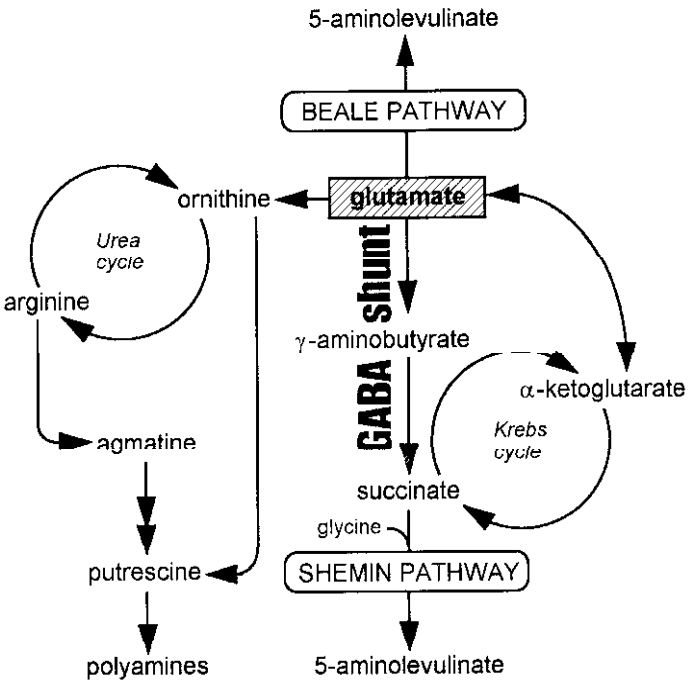

Fig. 1. Putative role of glutamate as a precursor of the Shemin pathway in a sugar beet callus grown under darkness. Relationships with the polyamine pathway, the GABA shunt and the Krebs cycle.

The Shemin pathway has never been reported in plant leaves (Beale 1990, Porra 1986) either by ALAS detection or by labelling experiments. The *in vitro* culture of sugar beet callus allowed us the detection of ALAS activity either under light or even more in darkness. This might be explained by a derepression of ALAS gene in heterotrophic conditions. Indeed, ALAS had also been detected in soybean cells (De Xifra *et al.* 1971) or callus (De Barreiro 1975) and in potatoes stored under dim light (Ramaswamy and Nair 1973). Moreover, our results show that glutamate or its derivatives might feed the Shemin pathway. Such an assumption agreed with experiments where in potato peelings the ^{14}C-glutamate was significantly incorporated into ALA via the Shemin pathway (Ramaswamy and Nair 1976). It remains to elucidate by labelling experiments the exact contribution of the GABA shunt and Krebs cycle to ALA synthesis in conditions preventing glutamate to be used through the Beale pathway.

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