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FORMATION OF PUTRESCINE AND CINNAMOYL PUTRESCINES IN TOBACCO CELL CULTURES

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Abstract—A *p*-fluorophenylalanine- (PFP) resistant cell line of *Nicotiana tabacum* and wild type cells accumulating high and low levels of cinnamoyl putrescines, respectively, were used to study the formation of putrescine in the biosynthesis of cinnamoyl putrescines. Labelled arginine and ornithine were equally well incorporated into the main conjugates caffeoyl and feruloyl putrescine. Trapping experiments indicated that both amino acids were decarboxylated for putrescine biosynthesis. Nearly all alcohol-extractable radioactivity from the labelled amino acids was found as cinnamoyl putrescines in the PFP-resistant cell line, whereas wild type cells retained significant radioactivity in the amino acids. The enzyme activities of arginine and ornithine decarboxylases in the resistant cell line were increased 3- to 6-fold.

INTRODUCTION

In mammalian cells, putrescine is only synthesized by L-ornithine decarboxylase (EC 4.1.1.17; ODC) which is also the rate-limiting enzyme for polyamine biosynthesis [1]. In higher plants, however, putrescine is usually formed by L-arginine decarboxylase (EC 4.1.1.19; ADC) via the intermediate, agmatine [2–4]. However, in the Solanaceae, putrescine is also formed by decarboxylation of L-ornithine [5–8]. In tobacco cell cultures and in apical parts of tobacco plants, putrescine is often conjugated with cinnamic acids [9, 10]. Recently, we have described a *p*-fluorophenylalanine- (PFP) resistant tobacco cell line (TX4) accumulating 10 times more cinnamoyl putrescines (e.g. caffeoyl and feruloyl putrescine) then wild type cells TX1 [11, 12]. The increased synthesis of the cinnamic acids was due to the higher activity of phenylalanine ammonia lyase (EC 4.3.1.5; PAL) in TX4 cells which was 10-fold greater than in the TX1 cells. Increased synthesis of the cinnamic acids was evidently accompanied by increased synthesis of putrescine for cinnamoyl putrescine biosynthesis. Therefore, a comparison of the formation of putrescine and cinnamoyl putrescines by high yielding TX4 cells with low yielding wild type cells was expected to give information about the participation of ODC and ADC in the biosynthesis of putrescine.

RESULTS AND DISCUSSION

From previous feeding experiments with phenylalanine it was known that TX1 and TX4 cells differ greatly in L-phenylalanine metabolism due to different capacity in synthesizing cinnamoyl putrescines [12, 13]. Therefore, precursors of the amine moiety of cinnamoyl putrescines were fed to TX1 and TX4 cells. After 6 hr, between 60 and 95% of ^{14}C-labelled putrescine, ornithine, and arginine was taken up by the cell lines. Up to 60% of label in arginine and 20–30% of label from putrescine or ornithine was not extracted by methanol–chloroform–water (MCW). The MCW extracts were chromatographed in various systems and scanned for distribution of radioactivity. After feeding of [^{14}C]-putrescine, [^{14}C]-ornithine and [^{14}C]-arginine to TX4 cells, *ca* 50% of the MCW-soluble radioactivity was always incorporated into caffeoyl and feruloyl putrescine (Table 1). In TX1 cells, most of the extractable radioactivity was still found as the given precursor (Table 1). Arginine and ornithine were equally efficient precursors of cinnamoyl putrescine biosynthesis. In TX1 cells the main metabolite of ornithine and arginine was putrescine. Since only a small percentage of the metabolized precursors was accumulated as putrescine in the TX4 cells, it is likely that the uncharacterized conjugating enzyme has a higher activity in TX4 cells than in TX1 cells.

It may be argued that arginase had converted arginine to ornithine which was then decarboxylated, or ornithine was converted first to arginine before being decarboxylated. Only small amounts of label from ornithine were found in free arginine (Table 1). Almost no label from arginine was found in ornithine. Moreover, this argument was completely refuted by adding 5×10^{-5} M arginine to wild type cells before feeding [^{14}C]-ornithine and vice versa. Such trapping had no influence on the amount of radioactivity found in putrescine while the label in the trapping amino acid was only slightly increased. Thus, both amino acids were directly decarboxylated for putrescine biosynthesis. This is in complete agreement with the formation of putrescine in tobacco leaf disks [5, 6]. By feeding [guanido-^{14}C]-arginine to the cells, it was shown that no cinnamoyl agmatines were formed as no label was detected in the phenolic conjugates.

The synthesis of cinnamoyl putrescines in tobacco cells was blocked by the competitive PAL inhibitor, α-aminooxy-β-phenylpropionic acid (AOP), and phenylalanine levels increased significantly [12]. Feeding of

Table 1. Metabolism of labelled precursors of cinnamoyl putrescines added to 5-day-old cell cultures of TX1 and TX4 for 6 hr

	Distribution of radioactivity in cell extracts in the absence and presence (in parentheses) of BAOP after feeding							
	[U-^{14}C]-L-phenylalanine		[1,4-^{14}C]-putrescine		[5-^{14}C]-L-ornithine		[U-^{14}C]-L-arginine	
	TX1	TX4	TX1	TX4	TX1	TX4	TX1	TX4
Cinnamoyl putrescines	12.6 (0.6)	60.8 (6.4)	19.8 (8.4)	61.5 (40.6)	12.0 (7.3)	43.3 (30.1)	16.2 (6.6)	45.5 (24.8)
Agmatine	—	—	—	—	—	—	5.1 (4.3)	2.3 (2.7)
Putrescine	—	—	63.2 (82.1)	18.8 (41.4)	16.4 (31.8)	9.2 (25.1)	21.5 (54.4)	18.7 (32.5)
Ornithine	—	—	—	—	56.0 (47.3)	24.8 (19.8)	—	—
Arginine	—	—	—	—	4.3 (6.8)	2.9 (6.6)	46.4 (34.6)	12.4 (14.6)
Phenylalanine	68.4 (88.3)	13.3 (78.2)	—	—	—	—	—	—

The per cent distribution was calculated from chromatograms of cell extracts in systems L1 and L2. The background radioactivity added to 100%. The effects of *N*-benzyloxycarbonyl-L-α-aminooxy-β-phenylpropionic acid (BAOP) on the metabolism of these labelled precursors are shown in parentheses. The cells were incubated for 3 hr with 2×10^{-5} M BAOP before the labelled compounds were added.

putrescine, ornithine and arginine, respectively, to TX1 and TX4 cells in the presence of an aminooxypropionic acid always resulted in accumulation of putrescine (Table 1). *N*-Benzyloxycarbonyl-L-α-aminooxy-β-phenylpropionic acid (BAOP) at 2×10^{-5} M blocked the conversion of phenylalanine to cinnamic acids almost completely (Table 1). The fact that putrescine conjugation with cinnamic acids was not similarly reduced (Table 1) indicated the availability of some free cinnamic acids for conjugation under the experimental conditions. The flow of ornithine and arginine into putrescine was not impaired when PAL activity was blocked by BAOP. Thus, depletion of cinnamic acids by the PAL inhibitor led to accumulation of putrescine.

Arginine and ornithine decarboxylase activities in extracts of TX1 and TX4 cells were also compared. Both enzymes were increased 3- to 6-fold in TX4 cells (Table 2). Since both enzyme activities were increased in the mutant line to a similar extent, and since both amino acids were directly decarboxylated during feeding experiments, the cells can evidently use either route to synthesize putrescine. Arginine decarboxylation was not inhibited by a 100-fold excess of ornithine, and vice versa proving that the enzymes were not competing for the same substrate (Table 3). The decarboxylases were inhibited by the corresponding amine: ADC by agmatine and ODC by putrescine. In animal cells two polyamine-regulatory mechanisms for ODC have been reported [14]. Compared to animal cells, however, rather high concentrations of putrescine had to be used for inhibiting ODC. Thus, it is not clear whether the polyamine levels were high enough for controlling the decarboxylation of the basic amino acids The levels of free putrescine vary during the growth cycle between 1.1–1.4 μmol/g dry wt in TX4 cells and 1.0–3.2 μmol in TX1. In both cell lines most of the synthesized putrescine is conjugated with cinnamic acids. TX4 cells accumulate more than 10% of its dry

Table 2. Specific enzyme activities of ornithine (ODC) and arginine (ADC) decarboxylase in TX4 cells and TX1 wild type cells

	Enzyme activity (nkat/g protein)	
	TX1	TX4
ODC 24 hr	0.75	2.84
120 hr	3.14	7.94
ADC 24 hr	0.50	3.65
120 hr	1.21	4.76

The enzymes were measured 24 and 120 hr after inoculation into fresh medium.

Table 3. Effects of some inhibitors on ornithine (ODC) and arginine (ADC) decarboxylase activity in protein extracts of TX4 cells

Inhibitor added [M]		Relative enzyme activity of ODC	ADC
None		100	100
Ornithine 10^{-3}		—	100
Arginine 10^{-3}		100	—
Putrescine 10^{-3}		58	88
Agmatine 10^{-3}		100	53
α-Difluoromethyl ornithine	10^{-3}	0	100
	5×10^{-5}	5	—
	10^{-5}	56	—
α-Methyl ornithine	10^{-3}	59	100
	10^{-4}	78	—

weight as cinnamoyl putrescines [11]. Nicotine is not formed in TX1 or TX4 cells.

The most effective inhibition of ODC was achieved with α-difluoromethyl ornithine, an enzyme-activated irreversible inhibitor of ODC. Concentrations between 5×10^{-5} and 10^{-4} M inhibited ODC completely, while ADC activity was not reduced, even by 10^{-3} M α-difluoromethyl ornithine (Table 3). The competitive inhibitor, α-methyl ornithine, reduced ODC activity only to 60% at 10^{-3} M. As in animal cells, α-difluoromethyl ornithine [15] was a very effective *in vitro* inhibitor of ODC of tobacco cells. However, in contrast to animal systems [15] 10^{-3} M α-difluoromethyl ornithine did not deplete the putrescine pool *in vivo* and thus cinnamoyl putrescine formation was not reduced. If ornithine decarboxylase was inhibited by α-difluoromethyl ornithine, the cells probably synthesized putrescine via the intermediate arginine.

The large accumulation of caffeoyl and feruloyl putrescine was only possible because the activities of the enzymes controlling the flow of primary metabolites into the secondary pathway were greatly increased in TX4. The comparison of TX1 and TX4 clearly showed that precursors for cinnamoyl putrescines were efficiently used only by cells which were competent for the synthesis of these secondary compounds.

EXPERIMENTAL

Plant material. Maintenance and some characteristics of wild type cells TX1 and PFP-resistant cells TX4 (*N. tabacum* L. cv Xanthi) have been described previously [11–13].

Feeding experiments. Cells (0.25 g) were inoculated into 20 ml of MS medium supplemented with 1.8 μM 2,4-D. The cells were grown for 7 days before incubation with the appropriate labelled compounds. The following labelled compounds were added: 1 μCi [U-^{14}C]-L-phenylalanine, [1,4-^{14}C]-putrescine, [5-^{14}C]-L-ornithine, [U-^{14}C]-L-arginine and [guanido-^{14}C]-L-arginine. The cells were harvested after the indicated period by filtration, freeze-dried, and extracted with MeOH–$CHCl_3$–H_2O (12:5:3) as described. The extracts were chromatographed with methylisobutylketone–HCO_2H–H_2O (14:3:2) on Si gel plates (L1) and with 1.5 M NaOAc on Polygram Ionex 25 SA-Na sheets (Macherey and Nagel) (L2), and scanned for radioactivity distribution. For quantitative measurement the corresponding zones were scraped off for scintillation counting. The residues of cell extraction were hydrolysed with 6 M HCl for 24 hr at 121° in an autoclave.

Enzyme prepn. Cells (5 g fr. wt) were ground in a chilled mortar in an ice bath with 2.5 g quartz sand, 1 g buffer satd Polyclar AT, and 5 ml buffer I (0.1 M Tris–HCl, pH 7.5, 10% glycerol, 15 mM 2-mercaptoethanol, 20 mM Na ascorbate, 5 mM EDTA, 1 mM pyridoxal phosphate). After centrifugation for 15 min at 15 000 g, the supernatant was treated with 2.5-fold vol. of a satd soln of $(NH_4)_2SO_4$ and stirred for 30 min before centrifugation. The ppt. was dissolved in 2.5 ml buffer II (0.1 M Tris–HCl, pH 7.5, 10% glycerol, 7.5 mM mercaptoethanol and 0.2 mM pyridoxal phosphate), layered on a prepacked PD10-Sephadex G 25-M column equilibrated with the same buffer. The enzymes were eluted with 3.5 ml buffer II as described by Pharmacia.

Enzyme assay. The standard incubation mixture for ODC contained 0.2 ml 2.5 mM [1-^{14}C]-L-ornithine (0.4 μCi), 0.3 ml 0.1 M Tris–HCl (pH 7.5) and 0.5 ml enzyme extract. The incubation was as described in ref. [16] in rubber-stoppered side-arm flasks (Kontes Glass Co.). The released $^{14}CO_2$ was trapped into 0.35 ml ethanolamine after adding 1 ml 1 N H_2SO_4 to the incubation mixture. Heat denatured enzyme extracts were used as blanks. For measurement of ADC the same concns were used. Protein was determined by the procedure in ref. [17]. Free putrescine was measured as its benzoyl derivative by HPLC [18].

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