Effects of α-Aminooxy-β-phenylpropionic Acid on Phenylalanine Metabolism in *p*-Fluorophenylalanine Sensitive and Resistant Tobacco Cells

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Z. Naturforsch. **34 c,** 770 – 775 (1979); received May 18, 1979

Nicotiana tabacum, Suspension Cultures, α-Aminooxy-β-phenylpropionic Acid, Chorismate Mutase, Phenylalanine, Pool Sizes

A *p*-fluorophenylalanine (PFP) resistant cell line with high phenylalanine ammonia lyase (PAL) activity and wild type cells with low PAL activity were compared in their responses to PAL inhibition by α-aminooxy-β-phenylpropionic acid (AOP). Inhibition of PAL reduced the levels of the main phenolic compounds to 30% of the controls. Free phenylalanine pools increased 17 fold in the resistant line and 6 fold in the sensitive line, respectively. The accumulation of phenylalanine did not reduce the flow of labeled shikimic acid into the aromatic amino acids tyrosine and phenylalanine. The results are discussed with respect to the feedback inhibition of chorismate mutase activity by phenylalanine and tyrosine in both cell lines.

Amino acid analog resistant cell lines normally have an increased pool size of the corresponding natural amino acid due to a lessened feedback control in the biosynthetic pathway [1]. A tobacco cell line resistant to *p*-fluorophenylalanine (PFP), however, did not fit into this frame [2–5]. The main reason for the resistance was ascribed to a decreased uptake of all L-amino acids [5]. Some alterations in the metabolism of phenylalanine may contribute to acquiring resistance, too. Phenylalanine ammonia lyase (PAL) activity of resistant cells was found to be 10 times the activity of sensitive cells [3]. This resulted in 6–10 times higher levels of phenolic compounds [4], of which more than 85% were identified as caffeoyl and feruloyl putrescine (unpublished). The pool sizes of all amino acids were found to be 3–5 times lower in the resistant strain (unpublished). The decreased pool size of phenylalanine in the resistant line did not fit with earlier observations [2] that chorismate mutase activity was less sensitive to feedback inhibition by phenylalanine and tyrosine.

To get more insight into the regulation of phenylalanine biosynthesis in cultured tobacco cells, we tried to eliminate the deamination of phenylalanine. Therefore the cells were grown in the presence of a specific inhibitor of PAL, α-aminooxy-β-phenylpropionic acid (AOP) [6]. Inhibition of PAL should result in distinct changes of phenylalanine pools in sensitive and resistant cells and/or should reduce the flow of carbon through the shikimate pathway.

Abbreviations: PFP, *p*-fluorophenylalanine; PAL, phenylalanine ammonia lyase; AOP, α-aminooxy-β-phenylpropionic acid; MCW, methanol : chloroform : water.

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0341-0382/79/0900-0770 $ 01.00/0

Materials and Methods

Plant material

Maintenance and characteristics of the sensitive cell line TX1 and the PFP-resistant line TX4 (*Nicotiana tabacum* L. cv. Xanthi) have been described previously [2–5]. For inhibition experiments with AOP 0.2 g fresh weight were inoculated in 20 ml of medium. AOP was added after sterile filtration at the beginning of the growth cycle or according to the following scheme: after 0 and 24 h 2×10^{-7} mol, 48 and 72 h 4×10^{-7} mol, 96 and 120 h 8×10^{-7} mol, 144 h 6×10^{-7} mol = $\Sigma\ 3.4\times10^{-6}$ mol/21.7 ml. This would yield a concentration of 1.57×10^{-4} M if given as one portion.

Feeding experiments

Cells grown in the absence or presence of AOP were incubated after 6 days of growth with either 1 μCi L-[U-^{14}C]-phenylalanine for 6 h or 0.5 μCi [2,3,4,5$_{(n)}$-^{14}C]-shikimic acid for 4 h. The evolution of $^{14}CO_2$ was measured as described [7].

Biochemical measurements

Freeze dried cells were extracted with MCW 12 : 5 : 3 and divided into a $CHCl_3$ and a CH_3OH : H_2O layer [4]. The water phase contained all important compounds for analysis. Phenolics were determined with the Folin reagent [4] or by the absorption at λ_{max} = 315 nm of the cinnamoyl putrescine derivatives. Amino acid pools were determined with an amino acid analyzer. If radioactive labeled compounds were fed to the cultures the water phases were chromatographed before and after separation on Dowex 50-H^+. Amino acids were eluted with 0.6 N NH_3. Chromatography was performed on TLC plates using the systems: I) methylisobutylketone : HCOOH : H_2O 14 : 3 : 2 (silica gel); II) *n*-butanol : CH_3COOH : H_2O 4 : 1 : 1 (cellulose); III) *n*-propanol : 30% NH_3 7 : 3 (cellulose); IV) ethyl acetate : methylethylketone : HCOOH : H_2O 5 : 3 : 1 : 1 (silica gel). Distribution of radioactivity was measured with a TLC-scanner and by scintillation counting of zones scraped off. Radioactivity in phenylalanine and tyrosine was also measured by collecting corresponding fractions of the amino acid analyzer eluate. Chorismate mutase was extracted and measured as described [8] with Sephadex G-25 filtration as additional step [3]. PAL activity was determined as before [3].

Results

Conditions for inhibition of PAL by AOP

Very low concentrations of AOP, added at the beginning of the growth cycle already reduced the levels of phenolic compounds in sensitive TX1 cells sharply. Somewhat higher concentrations of AOP were necessary to see a pronounced effect on the content of phenolics in resistant TX4 cells (Fig. 1). The loss in phenolics was accompanied by a slight increase in growth rates. The different sensitivity of cells with high (TX4) and low (TX1) PAL activity suggested that AOP may be more rapidly metabolized in TX4 cells, or that more AOP was necessary to inhibit the higher PAL activity in TX4 cells. To test this, TX1 and TX4 cells were incubated with [^{14}C]-phenylalanine in the presence of AOP (Table I). In TX1 cells a single dose of 10^{-5} M AOP reduced the flow of phenylalanine into phenolics nearly completely. In TX4 cells, however, the same dosage allowed

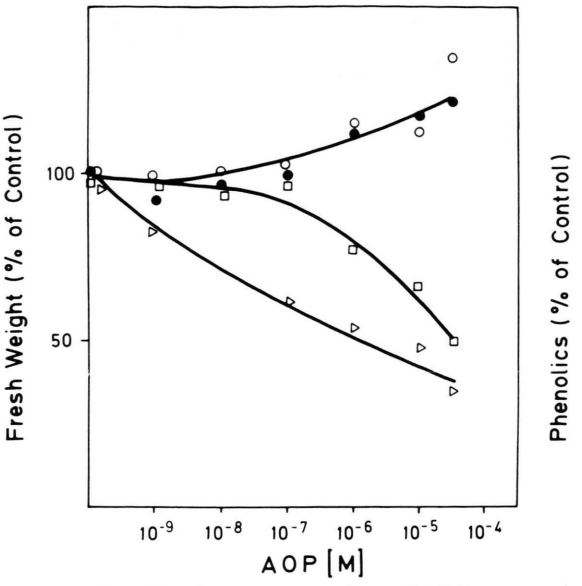

Fig. 1. Effect of various concentrations of AOP on growth (○---○ = TX1; ●---● = TX4) and phenolics (△---△ = TX1; □---□ = TX4).

Table I. Effect of AOP on the incorporation of [^{14}C]-phenylalanine into phenolics. Cells were grown without AOP, with a single dose of 10^{-5} M AOP or with 2 doses of 10^{-5} M AOP, the second given 3 h before incubation with 1 μCi [U-^{14}C]-phenylalanine for 6 h. Distribution of radioactivity in MCW-extracts was measured after TLC-chromatography in system I [4].

	AOP [M]	Distribution of radioactivity [%]	
		Phenolics	Phenylalanine
TX1	none	30	46
	10^{-5}	6.6	83
	$10^{-5}+10^{-5}$	2.7	97
TX4	none	70	16.6
	10^{-5}	53	33
	$10^{-5}+10^{-5}$	8	76

50% incorporation into phenolics. This flow was completely stopped by a second dosage of 10^{-5} M AOP given 3 h before incubation with [^{14}C]-phenylalanine. A complete inhibition of PAL during a whole growth cycle can only be acchieved by continuous supply of AOP to the resistant cell line.

Changes of levels of amino acids and phenolics by continuous doses of AOP

AOP was added to the cultures according to the scheme over the whole growth period. This continu-

Table II. Effects of AOP on growth rates, total phenolics and L-amino acids. AOP was continuously added according to the scheme (Material and Methods). Phenolics are expressed as equivalents of chlorogenic acid.

	TX1		TX4	
AOP	minus	plus	minus	plus
	[mg dry weight]			
growth	237 ∓ 22	140 ∓ 10	190 ∓ 10	155 ∓ 15
	[μmol/100 mg dry weight]			
phenolics	1.5 ∓0.1	0.5 ∓0.04	15.2 ∓0.1	5.3 ∓0.1
phenylalanine	0.73 ∓ 0.08	4.12 ∓ 0.36	0.24 ∓ 0.01	3.96 ∓ 0.28
tyrosine	1.94 ∓ 0.02	1.29 ∓ 0.10	1.01 ∓ 0.18	1.56 ∓ 0.20
valine	1.43 ∓ 0.01	0.66 ∓ 0.08	0.31 ∓ 0.10	0.58 ∓ 0.02
leucine	1.25 ∓ 0.01	1.08 ∓ 0.58	0.38 ∓ 0.08	1.10 ∓ 0.04

ous supply of AOP significantly reduced the increase in dry weight (Table II). The levels of cinnamoyl putrescine derivatives were diminished to 30% of the controls. The inoculum size could barely account for 10% of total phenolics. Thus, even the continuous supply of AOP did not completely block PAL activity in either cell line. The PAL inhibition led to a 6 fold increase in free phenylalanine in TX1 cells (Table II). Due to the high PAL activity the inhibition caused even a 17 fold rise in free phenylalanine in resistant TX4 cells. Extractable PAL activities remained unchanged in AOP treated and untreated TX4 cells. Some changes were found for other amino acids, too. However, the alterations of pool sizes were never as striking as for phenylalanine (Table II).

Feeding of [^{14}C]-shikimic acid

The increase in free phenylalanine by the inhibition of PAL should effect the flow of carbon through the shikimic acid pathway if pool sizes are under strict regulatory control. Therefore [^{14}C]-shikimic acid was fed to the continuously AOP treated cells (Table III). The poor uptake of shikimic acid by the resistant line was noted before [5]. Evolution of $^{14}CO_2$ was low as one would expect from the distribution of label in the shikimic acid. When [U-^{14}C]-shikimic acid was fed to the cultures 16% of the radioactivity taken up was found in CO_2 (Berlin and Widholm, unpublished) indicating that most of the shikimic acid taken up entered the shikimate pathway and was decarboxylated at the intermediate prephenate. Percent incorporation of radioactivity into protein and MCW-soluble compounds was similar in both cell lines (Table III). The chromatographic analysis of the MCW-extracts revealed the distribution given in Table IV. The incorporation of shikimic acid into phenolics was effectively blocked by the AOP treatment. Due to the PAL inhibition the ratio free tyrosine : free phenylalanine changed from 2.5 : 1 to 1 : 3 in TX1 cells (Table II) causing a relative increase of phenylalanine by a factor of 7.5 (TX4 cells: factor 10). These changes in pool sizes were not truly reflected by a corresponding distribution of radioactivity in tyrosine and phenylalanine (Table IV). This may be due to the relatively short feeding period of 4 h. Only 5–7% of the radioactivity were recovered as shikimic acid in AOP treated and untreated cells. The increased pool sizes of phenylalanine did not impede the flow of shikimic acid into the aromatic amino acids.

Chorismate mutase

An unchanged flow of shikimic acid would be reasonable if no control enzyme is between shikimic acid and L-phenylalanine/L-tyrosine. However, as

Table III. Distribution of radioactivity after feeding [2,3,4,5$_{(n)}$-^{14}C]-shikimic acid to TX1 and TX4 cells for 4 h. AOP was continuously added (Material and Methods). The numbers in brackets denote percent incorporation (percentage of radioactivity taken up).

	Distribution of radioactivity			
	TX1		TX4	
AOP	minus	plus	minus	plus
medium	8.3	10.8	67.4	65.7
CO_2	1.5 (1.6)	2.1 (2.4)	0.5 (1.5)	0.4 (1.2)
MCW-extract	43.6 (47.5)	39.0 (43.7)	14.2 (43.6)	11.0 (32.1)
protein	46.6 (50.8)	48.1 (53.9)	17.9 (54.9)	22.9 (66.8)

Table IV. Percent distribution of radioactivity of the MCW-extracts after feeding [^{14}C]-shikimic acid for 4 h. Chromatography in solvent I revealed the percent incorporation in tyrosine+phenylalanine and in shikimic acid+cinnamoyl putrescine derivatives. Chromatography of the MCW-extract after separation on a Dowex 50-H$^+$ column yielded the percent incorporation in shikimic acid (solvents II and IV) and in tyrosine and phenylalanine (solvent III). Cinnamoyl derivatives mainly remained on the Dowex column. The data in brackets denote the percent incorporation calculated from the collected fractions of the amino acid analyzer eluate.

	Distribution of radioactivity [%]			
	TX1		TX4	
AOP	minus	plus	minus	plus
shikimic acid	7.3	5.0	7.3	6.7
tyrosine	38.8 (27.3)	20.2 (20.4)	9.3 (15.2)	50.7 (48.9)
phenylalanine	26.4 (19.4)	60.1 (61.3)	32.3 (15.4)	30.3 (29.2)
phenolics	15.9	0.1	39.7	2.1

found before [2], chorismate mutase activity of tobacco cells was inhibited by low concentrations of L-phenylalanine (Fig. 2) and L-tyrosine. Generally chorismate mutase activity of sensitive cells was slightly more sensitive to feedback inhibition by phenylalanine. However, especially during the logarithmic growth phase the inhibition patterns were so similar in both cell lines that at least from crude extracts no conclusion could be drawn for a different feedback control.

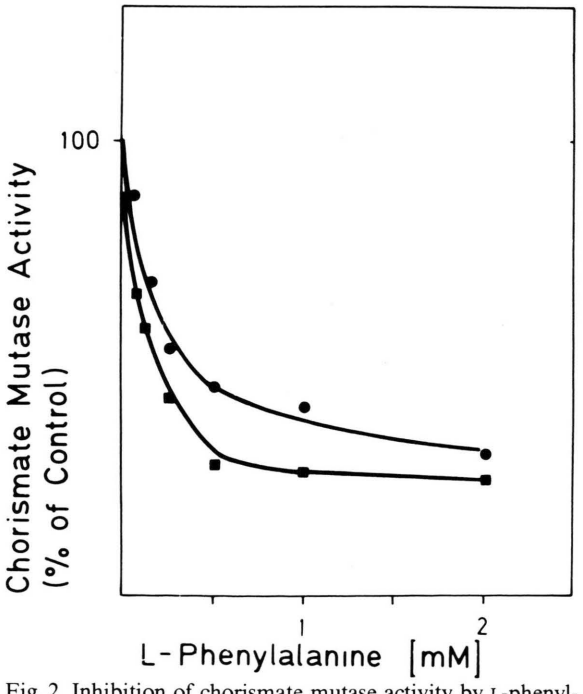

Fig. 2 Inhibition of chorismate mutase activity by L-phenylalanine in TX1 cells (■---■) and TX4 cells (●---●). When no inhibitor was present chorismate mutase produced 123 nmol prephenate/mg protein in TX1 cells and 146 nmol in TX4 cells per 30 min.

Discussion

The difference in phenylalanine metabolism between TX1 and TX4 cells arises from the different levels of PAL activity in these cells [3]. This difference was partly eliminated by adding the competitive PAL inhibitor AOP to the cell lines. The flow of phenylalanine into hydroxycinnamic acids was efficiently blocked after the application of AOP at least for certain time periods. However, under all conditions tested complete elimination of the PAL action could not be acchieved. The levels of cinnamoyl putrescine derivatives were reduced to 30% of the controls in both cell lines. Thus, TX4 cells continuously treated with AOP still contained 3 times more phenolics than untreated TX1 cells (Table II). Since the inhibitor was evidently more rapidly inactivated by TX4 cells it seems likely that AOP may be degraded by the action of PAL. Our experiments do not exclude that only the number of PAL molecules in TX4 cells was so high that the AOP concentrations given could not inactivate all enzyme activity. For long term experiments in systems with high PAL activity a continuous supply of AOP has to be guaranteed for maximal PAL inhibition.

The decrease of phenolics was accompanied by an increase in fresh weight (Fig. 1). A similar phenomon was described for Avena coleoptiles [9]. When PAL inhibition by PFP caused a depletion of the chlorogenic acid pool this was always accompanied by a segment elongation of Avena coleoptiles. AOP given continuously, however, reduced the growth

rate of tobacco cells (Table II) despite the lower levels of phenolics.

Inhibition of PAL by AOP is competitive and reversible, and inhibition of phenolic (anthocyanin) synthesis can be reversed by exogenous phenylalanine [6]. The increased pool sizes of phenylalanine by the AOP treatment, however, were not sufficient to lift the inhibition of PAL. The different increases in free phenylalanine favourably reflected the different levels in PAL activity in TX1 and TX4 cells. The pool sizes were altered in the lines by a biochemical manipulation. If amino acid analog resistant cells were selected showing a 6–7 fold increase in the corresponding amino acid concentration, the overproduction and hence the resistance were normally accounted to an altered feedback control in the biosynthetic pathway [1]. However, altered feedback inhibition is only one possible reason for increased pool sizes. As shown in this work the inhibition of a diverting pathway can contribute equally well to the observed phenomenon of increased amino acid pools.

Regulatory forms of chorismate mutase have been identified in a number of plants (see for ref. [10]). The *in vitro* results suggest that chorismate mutase of tobacco cells is a regulatory enzyme, too. By adding high concentrations of aromatic amino acids to cultured cells [11] or intact chloroplasts [12] it was shown that feedback inhibition may function *in vivo,* too. The main question, however, is whether the physiological pool sizes are high enough to make the enzyme act as control enzyme. Two observations let us conclude that the levels of free amino acids per se are hardly providing any information about the degree of regulatory controls. The two lines have chorismate mutases which are comparably sensitive to the feedback inhibitors. Nevertheless the pool sizes of phenylalanine in untreated TX1 and TX4 cells differ by a factor three (Table II). This lessened pool size in the resistant cell line cannot be explained by the increased level of PAL activity diverting phenylalanine into secondary pathways, since all free amino acid pools were found in lower concentrations. A more general control rather than a single control enzyme seems to be responsible for the levels of free amino acids in cultured cells. Free phenylalanine was increased manifold in tobacco cells by the AOP treatment. However, these higher amounts did not entail an inhibition of chorismate mutase activity. The flow of shikimic acid into the aromatic amino acids was not impeded by the enhanced level of phenylalanine. No intensified accumulation of shikimic acid was observed in AOP treated cells demonstrating that the accumulated amino acid did not act as feedback inhibitor. There may be two explanations for this: 1. The internal pool sizes are still too low to make them to feedback inhibitors. 2. The amino acids are immediately transported to other compartments so they cannot act as feedback inhibitors. It was shown that up to 70% of free amino acids were located in vacuoles of cultured carrot cells [13]. From this observation and the above presented data the existence of metabolically active and inactive pools seems to be possible in the tobacco cell lines. A similar compartmentation has been reported previously [14–16].

The rate of phenolic biosynthesis in tobacco cells and other systems is correlated with the level of PAL activity. However, phenolic biosynthesis seems to be independent from the pool size of phenylalanine [17, 18]. The results with variant cell lines of tobacco do not support the idea of a control at the level of substrate supply as postulated by Margna [19]. Whether further work with variant cell lines may give clues about regulatory connections between primary and secondary metabolism remains to see [20].

Acknowledgements

Financial support by Deutsche Forschungsgemeinschaft is gratefully acknowledged. We are especially thankful to Prof. Dr. N. Amrhein, Bochum, for a generous gift of AOP and valuable comments on the manuscript. We are indebted to Dr. M. R. Kula and Mrs. S. Löslein, GBF, Braunschweig, for running the amino acid analyses.

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