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Minireview

The control of L-phenylalanine ammonia-lyase activity by phosphonate and aminooxy analogues of phenylalanine

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L-Phenylalanine ammonia-lyase (PAL[1]; EC 4.1.3.5) which catalyses deamination of L-phenylalanine to *trans*-cinnamic acid, is a key enzyme in the secondary metabolism of plants. It supplies C_6–C_3 precursors for a large number of compounds, especially phenolics [1]. These compounds belong to a group of the so-called "secondary metabolites" although many of them play regulatory and metabolic functions in plants [2]. The enzyme was detected in higher plants as well as in microorganisms [1].

The physiology and biochemistry of this enzyme have been studied extensively [3]. In studies aimed at elucidation physiological functions of PAL special attention was paid to specific inhibitors of the enzyme.

In this minireview the emphasis is put on the comparison of the influence of putative inhibitors of PAL, aminooxy (α-aminooxy-β-phenylpropionic acid, AOPP) and phosphonic (1-amino-2-phenylethylphosphonic acid, PheP) analogues of phenylalanine. Amrhein & Gödecke [4] introduced L-AOPP as a potent inhibitor of phenylalanine deamination and proved that this compound, in micromolar or nanomolar concentrations, was a competitive inhibitor of PAL *in vitro*.

L-AOPP inhibited PAL from buckwheat (*Fagopyrum esculentum* Moench.) and from cell suspension cultures of carrot (*Daucus carota* L.) with K_i values of 1.4 nM and 2.4 nM, respectively [5]; K_i of 55 nM was determined for PAL from *Rhodotorula glutinis* [4].

Both optical enantiomers of AOPP were found to be inhibitory for PAL. Nevertheless, L-AOPP was generally a 10 - 20 times more efficient PAL inhibitor than the D-enantiomer [4]. It has been suggested that optical enantiomers of AOPP fit into the active site(s) of PAL in a mirror image relationship and act as transition state analogues in the elimination reaction [6].

PAL was irreversibly inhibited by L-AOPP [7] in cell suspension cultures of soybean (*Glycine max* (L.) Merr.); this can be explained, probably, by slow dissociation of the enzyme-ligand complex [8].

L-AOPP has been applied with considerable success in studies on regulation of the level of PAL in plant tissues [9 - 11], on synthesis of sphagnorubin [12], on lignin formation in xylem vessels [13], as well as in studies on the role of the products of PAL activity in the regulation of cell elongation [14], and of phytoalexin accumulation in pathogen resistance in soybean [15]. Generally, for effective suppression of phenylpropanoid synthesis *in vivo*, rather high concentrations of L-AOPP, i.e. ≥ 0.1 mM, have to be applied.

Another promising inhibitor of PAL of the group of phenylalanine analogues, is the phosphonous analogue, PheP. Aminophosphonic acids usually act as structural antagonists of amino acids and compete with their carboxylic counterparts for the active sites of enzymes or other cell receptors [16]. Those compounds are broadly defined as analogues of amino acids in which a carboxyl group has been replaced by a phosphonic group or a related one, e.g. phosphonous, phosphinic, etc. [16].

[1]Abbreviations: AOPP, α-aminooxy-β-phenylpropionic acid; PAL, L-phenylalanine ammonia-lyase; PheP, 1-amino-2-phenylethylphosphonic acid.

In the case of PAL from slices of potato tuber (*Solanum tuberosum* L.) PheP was less efficient in comparison with AOPP, as the apparent K_i values for L-PheP and D-PheP were found to be 6.5 μM and 3.3 - 5.3 μM, respectively [17]. It is intriguing that no single mechanism of an inhibitory action can be ascribed to PheP [17]. L-PheP was more effective than D-PheP. It inhibited PAL from the potato tuber either competitively at low concentrations, or the inhibition was mainly of the mixed type, or it was even uncompetitive at higher PheP levels [18].

PheP was a competitive inhibitor of buckwheat PAL activity. Both enantiomers were inhibitory but the L-enantiomer had higher affinity (K_i = 1.5 μM) for PAL than the D-enantiomer (K_i = 11.6 μM) [18].

It seems that other phosphonic analogues of Phe with alkyl chains by one methylene group longer or shorter than PheP, are also potent inhibitors of PAL activity, i.e. 1-amino-3-phenylpropylphosphonic acid (unpublished).

Non-acidic, or weakly acidic analogues of Phe, such as its amide or methyl esters, the tetrazole or methylphosphinic analogues, or phenylethylamine, were not, or only slightly, inhibitory; this clearly showed that the carboxyl group or its equivalent was required for binding of a ligand by PAL [18].

L-AOPP caused a large increase ("superinduction") in the extractable PAL activity in gherkin hypocotyls [9] and in cell suspension cultures of carrot [5]. By an immunoprecipitation technique, evidence was obtained that the increase in PAL activity in this instance resulted from the *de novo* enzyme synthesis [10, 11]. L-AOPP seems to be a specific inhibitor since it affects neither growth nor soluble protein content in suspension culture of *Daucus carota* L. [5]. Cinnamic acid which was also thought to be an inhibitor of PAL [2] alleviated this "superinduction" of PAL activity caused by L-AOPP in gherkin hypocotyls. The results reported by Lamb & Rubery [19] suggest that PAL may be regulated by *trans*-cinnamic acid. The mechanism proposed to explain the effects of L-AOPP *in vivo* involved modulation by cinnamate of a post-transcriptional stage of PAL formation [2].

In suspension cultures of carrot in presence of L-AOPP phenylalanine was accumulated while anthocyanin accumulation was inhibited [5]. Both enantiomers of AOPP inhibited anthocyanin formation in developing flowers of *Ipomea tricolor* Cav. and *Catharanthus roseus* Don. as well as in the seedlings of *Brassica oleracea* L. var. *capitata* (red cabbage), *B. oleracea* L. var. *caulo-rapa* (kohlrabi), with little interference with development of these tissues. Kohlrabi seedlings tolerated up to 0.3 mM L-AOPP without a reduction in fresh weight or chlorophyll content, while anthocyanin content was reduced by about 80% [20].

The biological activity of L-AOPP, as well as of other aminooxy compounds, is completely abolished in the presence of carbonyl compounds, such as pyruvate or acetone [18]. Using [2-^{14}C]acetone, L-AOPP can conveniently be quantitated in the assay originally devised for quantitation of a naturally occurring aminooxy compound, L-canaline (2-amino-4-(aminooxy)-butyric acid) [18]. Similar reactions with cellular metabolites might thus have decreased the concentration of L-AOPP in a tissue and reduced its efficiency as the PAL inhibitor.

It has been suggested that AOPP is not specific in its inhibitory action against PAL. Other biosynthethic pathways, for example ethylene synthesis, were also inhibited by AOPP at high concentrations [21]. AOPP used in studies *in vivo* at concentrations appropriate for PAL inhibition could, depending on the species, also inhibit tyrosine decarboxylase (EC 4.1.1.25) from *Syringa vulgaris* L. cell cultures and from *Hordeum vulgare* L. seedlings [23].

PheP was reported to inhibit competitively the activity of phenylalanyl-tRNA synthetases from *Aesculus hippocastanum* L. and *Aesculus parviflora* L. [22]. It is suprising that other aminoacyl t-RNA synthetases of *A. hippocastanum* were insensitive to phosphonate analogues of their respective natural amino acid substrates [23]. PheP either activated or inhibited pyruvate kinase from rabbit muscles [24].

PheP has been applied in studies on glyceollin accumulation and expression of physiological resistance to *Phytophtora megasperma* f.sp. *glycinea* in soybean [25]. In contrast to L-AOPP, L-PheP was not toxic to the zoospores which remained virulent in the presence of the inhibitor [25].

L-PheP (0.1 mM) did not reduce the division rate during the initial 3 - 4 days of culture of *Spirodela oligorrhiza* Kurz. (Hegelm.). Growth was more reduced by L-PheP than by its D-enantiomer [26].

There is evidence that PAL in *Robinia pseudoaccacia* L., *H. vulgare* L. and *Helianthus annuus* L. induced rythmic illumination-dependent changes in the activity [27 - 29]. A similar phenomenon was observed in duckweeds *Lemna perpusilla* and *Spirodela polyrrhiza* [30] as well as *S. oligorrhiza* Kurz. (Hegelm.) [31]. This periodicity did not occur in the presence of L-PheP [31].

PheP *in vivo* caused a "superinduction" of PAL activity in *S. oligorrhiza* [31], *Amaranthus caudatus* L. [32] and *Allium cepa* L. [33]. It seems that PheP stimulated PAL activity in short-term experiments *in vivo*, probably due to the phenomenon of superinduction described for AOPP [9].

L-PheP (50 μM) inhibited by 50% the light-induced anthocyanin synthesis in buckwheat hypocotyls, while the I_{50} value was nearly 20-fold higher in the case of the D-enantiomer [18]. The reduction of anthocyanin content was proportional to the potency of the analogues to inhibit the activity of buckwheat PAL *in vitro* [18].

The level of free phenylalanine was increased in the presence of PheP [17, 33, 34]. The authors suggested that phenylalanine which accumulated in the tissue treated with D,L-PheP originated from synthesis *de novo* because the accumulation of phenylalanine was suppressed by [*N*-(phosphonomethyl) glycine] (glyphosate), an inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EC 2.5.1.19) [18].

PheP increased PAL activity in *Spirodela* by about 32% whereas radioactivity in the PAL fraction was increased by 58%. Radioactivity of total proteins was increased by 32%. This means that PheP stimulated preferentially incorporation of the label into PAL [33]. Despite this, PAL activity under the influence of PheP *in vivo* was reduced [17, 18]. It is probable that PheP formed an inactive labile complex with PAL which dissociated upon extraction releasing the active enzyme. On adaptation to the inhibitory action of PheP and decrease in *trans*-cinnamate contents the plants accelerated synthesis of PAL. Synthesis of other proteins was also stimulated [33]. This led to accumulation of the PAL-PheP complex(es) and to "superinduction" of PAL when the activity was measured *in vitro*.

It seems that both PheP and AOPP influence plant cells in the same way. Contrary to conditions *in vitro*, the activities of the two compounds *in vivo* were comparable, may be due to the inactivation of AOPP by e.g. carbonyl compounds present in plant cells.

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