1-AMINO-2-PHENYLETHYLPHOSPHONIC ACID: AN INHIBITOR OF
L-PHENYLALANINE AMMONIA-LYASE IN VITRO**

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L(-)-, and D(+) enantiomers of 1-amino-2-phenylethylphosphonic acid (PheP),
a phosphonic analogue of phenylalanine, inhibit the activity of L-phenylalanine
ammonia-lyase (EC 4.3.1.5) of potato tuber tissue in vitro. The apparent type
of inhibition depends on concentration of PheP; as the concentration of D-PheP
is raised from 10⁻⁵ M to 2.5 x 10⁻³ M, the type of inhibition shifts from competitive
through mixed and non-competitive to uncompetitive. L-PheP exerts either
a competitive or mixed-type inhibition at low (10⁻⁶ - 10⁻⁸ M) or moderate
(5 x 10⁻⁵ - 2 x 10⁻⁴ M) concentration. Kᵢ for the concentration range of com-
petitive inhibition were 6.5 x 10⁻⁹ M, 5.3 x 10⁻⁵ M and 1.6 x 10⁻³ M for L-, D-,
and D.L-PheP, respectively. These Kᵢ values are valid for a relatively narrow
range of L-Phe concentration (0.2 - 4 mM) as L-phenylalanine ammonia-lyase
does not follow the Michaelis-Menten kinetics of the reaction.

Deamination of L-phenylalanine by L-phenylalanine ammonia-lyase¹
(EC 4.3.1.5) to trans-cinnamic acid, one of the precursors of phenyl-
propanoids: phenolics, flavonoids and lignin precursors, has been extensively
studied (Camm & Towers, 1973; Hanson & Havir, 1981; Jones, 1984). One line

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** This study was supported by the Ministry of Science, Higher Education and Technology
under the project R. 1.9.02. A preliminary report of the results was presented at the
XX Meeting of the Polish Biochemical Society, Olsztyn, September 18 - 20, 1984.

¹ Abbreviations used: AOPP, 2-aminoxy-3-phenylpropionic acid; PAL, L-phenylalanine
ammonia-lyase: Phe, L-phenylalanine: PheP, 1-amino-2-phenylethylphosphonic acid (phosphonium-
-phenylalanine).
of experimental work, aimed at elucidating details of the structure of the enzyme moiety and the function of its catalytic center(s), has been focused on characterization of natural and synthetic inhibitors of the enzyme and their mode of action. Application of more or less specific inhibitors reveals intrinsic details of physiological functions of the enzyme in vivo, especially control mechanisms of the enzyme activity and its role in plant development, differentiation and xylogenesis (Jones, 1984). The use of D-phenylalanine and benzoic acid in vitro showed that PAL, composed of two functional protomers, is unique among the allosteric enzymes since it does not follow either the classical allosteric model of Monod or the simple sequential model of Koshland, but exhibits a partially-concerted mechanism of subunit interactions (Nari et al., 1974).

Among natural compounds, cinnamic acid and its hydroxylated derivatives were found to be the most powerful inhibitors of PAL both in vivo and in vitro (Camm & Towers, 1973; Sato et al., 1982), and L-2-aminoxy-3-phenylpropionic acid, a hydroxylated analogue of phenylalanine, proved to be the most effective inhibitor in vitro (Amrhein & Gödeke, 1977). This suggested that other structural analogues of Phe might be also active against PAL. We have found that 1-aminopropylphosphonic acid, i.e. a phosphonic analogue of Phe (Scheme 1) is a competitive inhibitor of PAL from potato tuber tissue. Until now PheP was reported to inhibit competitively the activity of phenylalanyltRNA synthetases from *Aesculus hippocastanum* and *A. parviflora* (Anderson & Fowden, 1970). Also, depending on concentration, PheP either activated or inhibited pyruvate kinase from rabbit muscles (Izbicka-Dimitrijević et al., 1981). Recently we have shown that PheP, at a concentration but little affecting growth, blocked almost completely the synthesis of red pigments in *Spirodela oligorhiza*, *Fagopyrum esculentum*, *Brassica oleracea* and other plant species (Knypl et al., 1984; Knypl & Janas, 1986). In the short-term experiments PheP enhanced PAL activity severalfold in vivo although it exerted an inhibitory effect in vitro (Knypl & Janas, 1986). In the long-term experiments PheP induced the synthesis of a unique ribonuclease in *S. oligorhiza*, which was a glycoprotein of molecular weight of about 60 000 (a preliminary report was presented at the XX Meeting of the Polish Biochemical Society, Olsztyn, Sept. 18 - 20, 1984).
MATERIALS AND METHODS

PAL extraction and activity assay. The enzyme from the 1-mm thick slices of potato tuber (Solanum tuberosum L.), purchased on a local market, was induced and extracted according to Havir & Hanson (1970). An ammonium sulphate (0.27 - 0.4 sat.) precipitate, solubilized in 0.1 M-Na-borate buffer, pH 8.7, and stored at -20°C, was used as the enzyme source. The specific activity of the enzyme was 3.4 mU x mg^-1 protein (Havir & Hanson, 1970).

The enzyme assay mixture contained in a total volume of 1.5 cm^3: 0.2 M-Na-borate buffer, pH 8.7 (0.5 cm^3), PAL extract (0.01 unit, 0.05 cm^3) and either 1.45 cm^3 of redistilled water (control samples) or the same volume of PheP solution of the concentration indicated. After 10 min incubation at 40°C, 0.5 cm^3 of l-Phe solution was added (at the concentration indicated) and the absorbance at 290 nm was measured (t_0). The increase of A_290 was monitored for 60 min every 10 min. Since the reaction was linear for at least 90 min, ΔA_290 over the initial 30 min (t_30 - t_0) was taken as a measure of PAL activity. Results of the inhibition tests were evaluated according to Cornish-Bowden (1974) and Eisenthal & Cornish-Bowden (1974).

Tests were performed in duplicates and repeated at least thrice with the use of three different PAL preparations and 2 - 3 lots of PheP. In most of the experiments the following PheP preparations were used: L-PheP: [α]_578^20 = -50.6° (c = 2.0, 1 M-NaOH); d-PheP: [α]_578^20 = +46.7° (c = 2.016, 1 M-NaOH); otherwise, additional information is given (cf Table 1).

Table 1

<table>
<thead>
<tr>
<th>Enantiomer of PheP</th>
<th>K_i</th>
<th>The type of PAL inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-</td>
<td>[α] = +46.7</td>
<td>3.3 x 10^{-4} M</td>
</tr>
<tr>
<td>d-</td>
<td>[α] = +49.9</td>
<td>5.3 x 10^{-4} M</td>
</tr>
<tr>
<td>d,l-  (racemate)</td>
<td>[α] = -50.6</td>
<td>1.6 x 10^{-5} M</td>
</tr>
<tr>
<td>l-</td>
<td>[α] = -50.6</td>
<td>6.5 x 10^{-6} M</td>
</tr>
</tbody>
</table>

Synthesis of PheP. Racemic PheP was synthesized according to the method described earlier (Kowalik et al., 1981). As shown in Scheme 2, the synthesis starts with diethyl 1-oxo-2-phenylethyl phosphonate (I) readily obtainable from phenylacetyl chloride and triethyl phosphonite. I was converted into oxime II which in turn was reduced in the zinc-formic acid system to give aminophosphonate III. Acid hydrolysis of III led to 1-amino-2-phenylethylphosphonic acid IV in 65% yield. Enantiomers of PheP
were fractionated by crystallization of the diastereomeric salts of III with dibenzoyl-L(+)tartaric acid (Kowalik et al., 1981, 1984).

Specific rotations of the purest preparations of (+) and (−) enantiomers of PheP were +50.6° and −50.6° (c = 2, 1 M-NaOH), respectively. X-ray examination of single crystals of optically active isomers has shown the S configuration for (+)PheP and R configuration for the (−)PheP enantiomer (Kowalik et al., 1983). Because of the priority rules the R configuration of (−)PheP corresponds with the L-configuration of natural phenylalanine.

\[
\begin{align*}
\text{Ph-CH}_2-\text{C-PO}_3\text{Et}_2 & \xrightarrow{\text{NH}_2\text{OH, EtOH}} \text{Ph-CH}_2-\text{C-PO}_3\text{Et}_2 \\
\text{II} & \xrightarrow{\text{Zn/HCOOH}} \text{II}
\end{align*}
\]

\[
\rightarrow \text{Ph-CH}_2-\text{CH-PO}_3\text{Et}_2 \xrightarrow{\text{H}_2\text{O}} \text{Ph-CH}_2-\text{OH-PO}_3\text{H}_2 \\
\text{III} & \xrightarrow{\text{NH}_2} \text{IV}
\]

Scheme 2. Synthesis of L-amino-2-phenylethylphosphonic acid.

Reagents. L-β-Phenylalanine, chromatographically homogeneous, was produced by Reanal (Budapest, Hungary); other chemicals (POCh, Gliwice, Poland) were of analytical grade.

RESULTS

The apparent \( K_m \) of L-phenylalanine ammonia-lyase from potato tubers increases from 0.038 mM to 0.26 mM as the concentration of L-phenylalanine is increased (Havir & Hanson, 1970). \( K_m \) of the enzyme preparation applied in this study was about 0.23 mM for 2 mM-Phe.

L-PheP was found to be a more efficient inhibitor of PAL than the D-enantiomer, at the Phe concentration applied (2 mM; Fig. 1). Tests performed with varying concentrations of both PheP and Phe revealed that D-PheP at low concentration of about \( 4 \times 10^{-5} \) M acted as competitive inhibitor of PAL (Fig. 2a). At higher concentration, from \( 8 \times 10^{-5} \) to \( 2 \times 10^{-4} \) M, it exerted a mixed type of inhibition of PAL activity (Fig. 2b). Non-competitive inhibition was observed at about \( 10^{-3} \) M (not shown), whereas at the highest concentration tested, i.e. \( 2.5 \times 10^{-3} \) M, D-PheP behaved like an uncompetitive inhibitor (Fig. 2c). Similar plots (Eisenthal &
Cornish-Bowden, 1974) made for L-PheP revealed that this enantiomer at a concentration of $10^{-6}$ to $10^{-4}$ M inhibited PAL competitively, whereas at a higher concentration it exerted a mixed type of inhibition (the highest L-PheP concentration tested was $4 \times 10^{-4}$ M), (not shown).

Plots of $S/V$ against $I$ indicated that, over the concentration range from $10^{-5}$ to $8 \times 10^{-5}$ M, D-PheP inhibited PAL activity competitively as inferred from the parallel course of the plots for different concentrations of L-phenylalanine (Fig. 3). Shifts in the type of inhibition of PAL activity at low and moderate concentrations of L-PheP as dependent on Phe concentration are shown in Fig. 4 which represents plots of $S/V$ against $I$ (Cornish-Bowden, 1974). This type of plot is complementary to the popular Dixon plot: $1/V$ versus $I$. If both plots are drawn from the same group of experimental data, the type of inhibition can unambiguously be determined since the values of $K_i$ (the dissociation constant of the EI complex) and $K'_i$ (the dissociation constant of the EIS complex) can be calculated. When $K'_i$ goes to infinity, the enzyme is inhibited competitively. This case is illustrated by parallel plots representing $S/V$ against $I$ for increasing concentrations of the substrate (Fig. 3; Fig. 4: plots for 2 and 4 mm-Phe). Intersection of the plots below the $I$ axis ($K_i < K'_i$) is characteristic for the mixed type of inhibition (Fig. 4, plots for 0.4, 0.8 and 2 mm-Phe). Data from Fig. 4 indicate that despite the fact that clearly expressed non-competitive inhibition of PAL by L-PheP was not detected by the plots constructed according to Eisenthal & Cornish-Bowden (1974), (cf Fig. 2), this type of inhibition seems to occur at L-Phe concentration below 0.4 mm. The effect of racemic PheP was intermediate between those of L- and D-PheP. The racemate inhibited competitively PAL activity when it was applied at concentrations from $10^{-5}$ to $8 \times 10^{-5}$ M (Fig. 5).
Fig. 2. Competitive (a), mixed (b), and uncompetitive (c) inhibition of PAL activity by D-PhcP. Concentrations of D-PhcP: a, $4 \times 10^{-5} \text{ M}$; b, $2 \times 10^{-4} \text{ M}$; c, $2.5 \times 10^{-3} \text{ M}$. Concentrations of L-PhcP (mM): 0.2, 0.4, 0.8, 2.0 and 4.0. The results are expressed as $A_{290nm}$ after 30 min reaction at 40°C.
Fig. 3. Competitive inhibition of PAL activity by D-PheP. Details as in Fig. 2.

Fig. 4. Plots of $S/v$ against $I$ showing competitive, mixed, and non-competitive inhibition of PAL activity by t-PheP.
Fig. 5. Plots of $1/v$ against $I$ (Dixon plot) and $V/S$ against $I$, showing competitive inhibition of PAL activity by D.L-PheP.

Values of $K_i$ for D-, L-, and D,L-PheP are presented in Table 1. It should be pointed out that $K_i$ values for two lots of D-PheP, varying in purity, were different.

The values of $K_i$ for PheP should be taken into consideration only as tentative data, in view of the fact that PAL does not follow the Michaelis-Menten kinetics (Nari et al., 1974; Hanson & Havir, 1981). Depending on the inhibitor concentration, the double reciprocal plots $1/v$ against $1/S$ were either concave downwards or concave upwards (Fig. 6 a-c). Nevertheless, in a narrow range of L-phenylalanine concentration, the plots could be regarded as straight. Due to this intentional simplification, PAL could be analysed by the methods applicable to “Michaelian-type enzymes” (cf Hanson & Havir, 1981), as it clearly shown in Figs. 2 - 5 and the data of Fig. 7, representing direct experimental values of $v$ versus $S$ as they had been recorded in typical experiments.
Fig. 6. Double reciprocal plots of the reaction catalysed by PAL in the presence of different concentrations of either D-PheP (a, lower concentrations and b, higher concentrations) or L-PheP (c).
DISCUSSION

L-AOPP (L-2-aminoxy-3-phenylpropionic acid) competitively inhibited PAL from various sources. $K_i$ values for PAL from *Fagopyrum esculentum* and *Rhodotorula glutinis* were reported to be 1.4 mM and 55 nM, respectively (Amrhein & Gödeke, 1977). The corresponding values for D-AOPP were about 20-fold higher (Amrhein & Holländer, 1979). In the case of PAL from

![Graph](image)

Fig. 7. Plots of $v$ versus $I$, as recorded in typical experiments on the inhibition of PAL activity by D- (a) and L- (b) enantiomers of PheP.

suspension cell cultures of *Daucus carota* L. and the seedlings of *Raphanus sativus* L., $K_i$ values for L-AOPP were 2.4 nM and 11.3 nM, respectively (Noé *et al.*, 1980; Noé & Seitz, 1982; Strack *et al.*, 1978). In contrast to the effects *in vitro*, L-AOPP induced *in vivo* PAL synthesis in gherkin hypocotyls (*Cucumis sativus* L.) and carrot cell suspension cultures, the
phenomenon named “superinduction” (Amrhein & Gerhardt, 1979; Noé & Seitz, 1982). A similar “superinduction” of PAL activity under the influence of PheP was noted in Spirodea oligorrhiza (Kurz) Hegelm. and Amaranthus caudatus L. (Knypl & Janas, 1986).

PheP seems to be much less efficient in comparison with AOPP, as the apparent $K_i$ values for L-PheP and d-PheP were found to be $6.5 \times 10^{-6}$ M and $3.3 - 5.3 \times 10^{-5}$ M, respectively. However, it is interesting that PheP acts like an apparently competitive inhibitor of PAL at low concentration or as a non-competitive inhibitor at high concentration, whereas over the whole range of moderate levels it exerts an apparently mixed type of inhibition. This relation is especially clear in the case of d-PheP which at the concentration of $2.5 \times 10^{-3}$ M inhibited PAL uncompetitively. L-PheP is more effective than d-PheP (Fig. 1) and it inhibits PAL from the potato tuber either competitively at low concentration, or the inhibition is mainly of the mixed type at higher L-PheP level. Hence, the L-enantiomer seems to be more specific than the d-enantiomer, although it can also exert a non-competitive inhibition at relatively low d-Phe concentrations (Fig. 4).

In the tests in vitro, PheP inhibited the activity of PAL from Zea mays L., Fagopyrum esculentum Moench., Cucumis sativus L., Spirodea oligorrhiza (Kurz) Hegelm., Amaranthus caudatus L., and other plant species (Knypl & Janas, 1986). In the experiments in vivo PheP doubled the activity of PAL in Spirodea oligorrhiza, and strongly inhibited synthesis of anthocyanins in red cabbage (Brassica oleracea L. var capitata) seedlings (Knypl & Janas, 1986). In the latter case the PAL activity was moderately reduced. Hence, the effect of PheP in vivo seems to be similar to that provoked by AOPP (Amrhein & Holländer, 1979).

This study was based on an intentional simplification, i.e. on the assumption that over a rather narrow range of L-phenylalanine concentration the enzyme follows the Michaelis-Menten kinetics. Validity of this assumption was justified by the experimental data (Figs. 1 - 5). Nevertheless, it is well documented that PAL, composed of four polypeptides, should be regarded as a molecule made of two functional protomers (each composed of one α and one β polypeptide). The enzyme, as a matter of fact, follows neither the classical allosteric model of Monod nor the simple sequential model of Koshland. Binding of the substrate induces a conformational change in the corresponding functional protomer as well as the appearance of a new conformation of the unliganded protomer (Nari et al., 1974; Hanson & Havir, 1981). More detailed studies should be performed to elucidate the molecular mechanism of inhibition of PAL activity by the optically active enantiomers of PheP, and to find a proper mathematical model explaining the true type of inhibition, tentatively called here “apparently” competitive, mixed, non-competitive or uncompetitive. Although the data presented in this report should be regarded as preliminary ones, they clearly demonstrate that the
two enantiomers of PheP offer a promising tool for studies both on structure and function of active site(s) of PAL, as well as the role this enzyme plays in vitro (cf Jones, 1984).

REFERENCES


Received 25 October, 1984:
revised 23 January, 1985