Phenylalanine Analogues: Potent Inhibitors of Phenylalanine Ammonia-Lyase Are Weak Inhibitors of Phenylalanine-tRNA Synthetases

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Dedicated to Professor Achim Trebst on the occasion of his 65th birthday

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(1-Amino-2-phenylethyl)phosphonic acid (APEP), (1-amino-2-phenylethyl)phosphonous acid (APEPi), α-aminooxy-β-phenylpropionic acid (AOPP) and several other phenylalanine analogues are potent inhibitors of (*S*)-phenylalanine ammonia-lyase (PAL) *in vitro* and *in vivo*. The ability of these compounds to inhibit (*S*)-phenylalanine-tRNA synthetases (PRSs) from wheat germ, soybean, and baker's yeast has been investigated and compared to the inhibition of PAL. APEP and APEPi were found to inhibit the $tRNA^{phe}$-aminoacylation reactions catalyzed by the three PRSs studied *in vitro* in a competitive manner with respect to (*S*)-phenylalanine. (*R*)-APEP inhibits the PRSs with apparent K_i values of 144 μM for wheat germ (app. K_m for (*S*)-phe 5.2 μM), 130 μM for soybean (app. K_m for (*S*)-phe 0.9 μM), and 1096 μM for baker's yeast (app. K_m for (*S*)-phe 5.5 μM). The apparent K_i values for (*R*)-APEPi are 315 μM, 160 μM, and 117 μM, respectively. APEP and APEPi inhibit the ATP-pyrophosphate exchange reactions catalyzed by the PRSs from wheat germ and baker's yeast, but they are not activated and do not serve as substrates in these reactions. AOPP has no affinity to any of the three PRSs, whereas it is a potent inhibitor of PAL. In light of our *in vitro* results with PRSs from different sources it appears unlikely that the PAL inhibitors we have studied have any significant inhibitory effect on this essential step in protein synthesis *in vivo*.

Introduction

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the formation of *trans*-cinnamic acid from (*S*)-phenylalanine, the first committed step in the biosynthesis of phenylpropanoid compounds (for a recent review, see Hahlbrock and Scheel, 1989).

Over many years we have screened phenylalanine analogues as putative specific inhibitors of PAL *in vitro* and *in vivo* (Amrhein, 1986). In 1977, we introduced the *O*-hydroxylamine analogue of (*S*)-phenylalanine, (*S*)-α-aminooxy-β-phenylpropionic acid ((*S*)-AOPP), as a potent inhibitor of this enzyme. (*S*)-AOPP inhibits PAL from buckwheat *(Fagopyrum esculentum) in vitro* in a competitive manner with respect to (*S*)-phenylalanine (K_m 45 μM) with an extremely low K_i of 1.4 nM (Amrhein and Gödeke, 1977). PAL from soybean was apparently irreversibly inhibited by (*S*)-AOPP (Havir, 1981; Jones and Northcote, 1984).

Later, we described the phosphonic analogue of (*S*)-phenylalanine, (*R*)-(1-amino-2-phenylethyl)-phosphonic acid ((*R*)-APEP), as another potent competitive inhibitor of PAL from buckwheat (Laber *et al.,* 1986). The buckwheat enzyme was inhibited by (*R*)-APEP with a K_i of 1.5 μM (Laber *et al.,* 1986). Likewise, the PAL from potato tubers was also inhibited competitively by (*R*)-APEP *in vitro* (Janas *et al.,* 1985).

In vivo inhibition of PAL by (*S*)-AOPP and (*R*)-APEP was demonstrated by their effect on the formation of phenylpropanoid compounds in plant tissues: they blocked the light-induced biosynthesis of anthocyanin and other phenylpropanoid

Abbreviations: AOPP, α-aminooxy-β-phenylpropionic acid; APEP, (1-amino-2-phenylethyl)phosphonic acid; APEPi, (1-amino-2-phenylethyl)phosphonous acid; BD cellulose, benzyolated DEAE cellulose; n.d., not determined; PAL, (*S*)-phenylalanine ammonia lyase; (*S*)-phe, (*S*)-phenylalanine; PP_i, pyrophosphate; PRS, (*S*)-phenylalanine-tRNA synthetase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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compounds in etiolated buckwheat hypocotyls (Amrhein and Gödeke, 1977; Laber *et al.*, 1986) and provoked the accumulation of phenylalanine (Laber *et al.*, 1986; Holländer *et al.*, 1979). Although the K_i of (*R*)-APEP for buckwheat PAL is higher by three orders of magnitude than that of (*S*)-AOPP, their *in vivo* effects are comparable at equimolar concentrations (Laber *et al.*, 1986).

While PAL was shown to be a target of both (*S*)-AOPP and (*R*)-APEP *in vivo,* these inhibitors were also shown to interfere with the activity of other enzymes. Thus, (*S*)-AOPP is a strong inhibitor of tyrosine decarboxylases of several plant species (Chapple *et al.*, 1986), and high concentrations of this compound affect ethylene biosynthesis (Amrhein and Wenker, 1979). On the other hand, AOPP and APEP inhibited a phenylalanine aminotransferase of mungbean only weakly (Laber *et al.*, 1986). APEP and the other phosphonic and phosphonous acid analogues described in this paper had no effect on another phenylalanine metabolizing enzyme, *viz.* phenylalanine-4-hydroxylase from rat liver (Zoń and Laber, 1988).

An important biochemical process of central function in all cells which might be inhibited *in vivo* by phenylalanine analogues is the biosynthesis of proteins. (*S*)-Phenylalanine-tRNA synthetase (PRS, EC 6.1.1.20), like other aminoacyl-tRNA synthetases, is highly selective in its recognition of both the amino acid to be activated and of the tRNA acceptor and thus ensures the fidelity of protein synthesis (Freist *et al.*, 1985). Like other aminoacyl-tRNA synthetases (amino acid-tRNA ligases, EC 6.1.1.–) (for reviews, see Joachimiak and Barciszewski, 1980; Lea and Norris, 1977; Lavrik and Moor, 1985) it catalyzes the transfer of a specific amino acid to its cognate tRNA in the tRNA-aminoacylation reaction. This reaction consists of two steps, i) a reversible activation of the amino acid to its adenylate, and ii) an irreversible transfer of the amino acid from the aminoacyl adenylate to its cognate tRNA, forming the aminoacyl-tRNA.

If PRS were unable to discriminate between its natural substrate, (*S*)-phenylalanine, and a phenylalanine analogue introduced into a cell, either inhibition of protein synthesis, or the synthesis of non- or malfunctioning proteins would ensue with the consequence of a general disruption of metabolism. Such an analogue would therefore be unsuitable as an inhibitor of a phenylalanine metabolizing enzyme *in vivo.* Due to the discriminating power of aminoacyl-tRNA synthetases, one may expect that few amino acid analogues will prove to be powerful inhibitors of their respective activating enzymes, but the prudent use of such inhibitors in metabolic studies requires knowledge of their possible interference with protein synthesis.

(*S*)-AOPP was previously shown not to inhibit PRS from *Phaseolus aureus* (Norris, 1975). In contrast, racemic APEP was found to be a strong competitive inhibitor of the PRS from horse chestnut *(Aesculus hippocastanum)* (see Table IV) and from *A. parviflora* in the (*S*)-phenylalanine-activation reaction (Anderson and Fowden, 1979a, b). (*R,S*)-APEP also inhibited the PRS from *E. coli* competitively in both the (*S*)-phenylalanine-activation reaction and the $tRNA^{phe}$-aminoacylation reaction (see Table IV) (Santi *et al.*, 1971). Surprisingly, the (*S*)-leucine- and (*S*)-threonine-tRNA synthetases from *A. hippocastanum,* and the (*S*)-tyrosine-tRNA synthetase from *A. californica* were totally insensitive towards the phosphonic acid analogues of their respective natural amino acid substrate (Anderson and Fowden, 1970a, b).

The aim of this work was to investigate the effect of several phenylalanine analogues on the initial step of phenylalanine incorporation into proteins. As *in vitro* test systems we chose the PRSs from wheat germ *(Triticum aestivum),* soybean *(Glycine max)* and baker's yeast *(Saccharomyces cerevisiae).*

Materials and Methods

Plant material

Wheat germ (type I) was purchased from Sigma, Munich (Germany). Soybean cultivar "Williams" was grown for 5 days at 25 °C in the dark.

Chemicals

$tRNA^{phe}$ and an aminoacyl-tRNA synthetase mixture, both from baker's yeast, were purchased from Sigma, Munich (Germany), and BD cellulose from Boehringer, Mannheim (Germany). (*S*)-[U-^{14}C]phenylalanine (19.4 MBq/μmol) and [^{32}P]-pyrophosphate (0.7 MBq/μmol) were purchased from Amersham-Buchler, Braunschweig (Ger-

many). The phosphonic and phosphonous phenylalanine analogues were provided by Dr. L. Maier (Ciba Geigy Ltd., Basel, Switzerland).

tRNA-aminoacylation assay

The assay for the wheat germ PRS contained in 100 µl: 50 mM Tris/HCl, pH 8.2, 15 mM $MgCl_2$, 50 mM KCl, 5 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, 2 mg/ml yeast total RNA, 4 mM ATP, 3 µM $tRNA^{phe}$ from wheat germ, 0.3 to 2 µM (*S*)-[^{14}C]phenylalanine (0.6 to 3.9 kBq). After 5 min preincubation, the reaction was started by addition of 0.06 pkat wheat germ PRS. The reaction was allowed to proceed for 6 min at 37 °C and was then stopped by addition of 0.2 ml ice-cold 10% (w/v) trichloroacetic acid. The mixture was then filtered under vacuum (glass fibre filter, Schleicher & Schuell No. 6). The filters were successively rinsed with ice-cold solutions of 10–15 ml 5% (w/v) trichloroacetic acid containing 5 mM phenylalanine, and of 5 ml 96% (v/v) ethanol containing 2% (w/v) potassium acetate. After air-drying, 5 ml Quickszint 212 (Zinsser, Frankfurt, Germany) were added and the radioactivity was measured in a liquid scintillation counter.

Aminoacylation reactions catalyzed by PRS from soybean and yeast were measured in the same way using $tRNA^{phe}$ from soybean and yeast, respectively. The pH in the PRS assays was 7.8 for soybean, and 7.4 for yeast. In contrast to the reaction mixture for wheat germ and soybean PRS, the mixture for the yeast enzyme contained 5 mM $MgCl_2$, 10 mM KCl and 2 mM ATP.

ATP pyrophosphate exchange assay

The activation of phenylalanine was measured using the procedure described by Carias and Julien (1976).

Isolation and purification of $tRNA^{phe}$

The isolation of tRNA from wheat germ and etiolated soybean cotyledons followed the procedure of Dudock *et al.* (1969). After DEAE cellulose column chromatography, $tRNA^{phe}$ of wheat germ was purified using BD cellulose chromatography according to method B described by Rafalski *et al.* (1977).

Purification of the PRS

PRS was extracted from 50 g wheat germ and purified by ammonium sulphate precipitation, gel filtration on Sephadex G-200 and hydroxyapatite chromatography as described by Carias and Julien (1976). Further purification was achieved by affinity chromatography. Using wheat germ tRNA, an anti-affinity tRNA-Sepharose lacking $tRNA^{phe}$, and an affinity $tRNA^{phe}$-Sepharose were prepared according to Swamy and Pillay (1979). Both columns were equilibrated in 50 mM acetate buffer, pH 5.5, containing 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM ethylenediaminetetraacetic acid, and 10% (v/v) glycerol. Elution from the anti-affinity column was performed with 0.1 M Tris/HCl, pH 8.0 containing the same constituents as the equilibration buffer. The pooled active fractions were applied onto the $tRNA^{phe}$-affinity column and elution was carried out in the same way. The (*S*)-phenylalanine affinity column was prepared according to Robert-Gero and Waller (1974). Chromatography was carried out as described by Schiller and Schlechter (1974). The equilibration buffer contained 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% (v/v) glycerol. For elution a linear gradient of KCl from 0 to 1.5 M in equilibration buffer was used.

Soybean PRS was extracted from 100 g of cotyledons and purified according to Swamy and Pillay (1980).

Results

Purification of the PRS

Using affinity chromatography, PRS from wheat germ *(Triticum aestivum)* was purified 223-fold to a specific activity of 324 pkat/mg (Table I). SDS-PAGE of the enzyme preparation revealed five major protein bands with molecular masses of 69.3, 61.2, 59.6, 55.0 and 38.4 kDa, respectively (data not shown).

From soybean *(Glycine max)* cotyledons, PRS (113 pkat) was purified 414-fold to a specific activity of 58 pkat/mg. SDS-PAGE of this enzyme preparation revealed three major protein bands with molecular masses of 78.5, 60.6 and 41.7 kDa (data not shown).

Table I. Purification of PRS from wheat germ *(Triticum aestivum)*.

Purification step	Protein [mg]	Activity [pkat]	Recovery [%]	Specific activity [pkat/mg]	Purification factor
Crude extract	6372	9217	100	1.5	1.0
$(NH_4)_2SO_4$ fraction (35–55%)	3231	7208	78.2	2.2	1.5
Sephadex G-200	2241	6870	74.8	3.1	2.1
Hydroxyapatite	1460	10921	7.5	118.5	5.2
Anti-tRNA Sepharose	1155	9067	98.4	7.9	5.4
$tRNA^{phe}$ Sepharose	80	5186	56.3	64.4	44.4
(*S*)-Phenylalanine Sepharose	13	4211	45.7	323.9	223.2

Enzyme kinetic data

Both Hanes and Lineweaver-Burk plots (Bisswanger, 1979) gave straight lines when PRS activity expressed as S/V and 1/V, respectively, from wheat germ *(Triticum aestivum)* was plotted against the (*S*)-phenylalanine concentration (S and 1/S, respectively) in both the $tRNA^{phe}$-aminoacylation (Fig. 1) and the ATP-PP_i exchange assays. From Hanes plots we calculated apparent K_m values for (*S*)-phenylalanine of 5.2 µM and 51 µM, respectively, in the two reactions. Using the statistical methods described by Wilkinson (1961), the same values were obtained from Lineweaver-Burk plots.

Table II summarizes the K_m values determined for the different substrates of the PRS from wheat germ, soybean cotyledons and baker's yeast. For the wheat germ enzyme we obtained a linear curve in the respective Hanes plot up to substrate concentrations of at least 10 µM (*S*)-phenylalanine (Fig. 1), 5 µM homologous $tRNA^{phe}$, and 5 mM ATP in the $tRNA^{phe}$-aminoacylation assay, and of 2 mM (*S*)-phenylalanine in the ATP-PP_i exchange assay (data not shown).

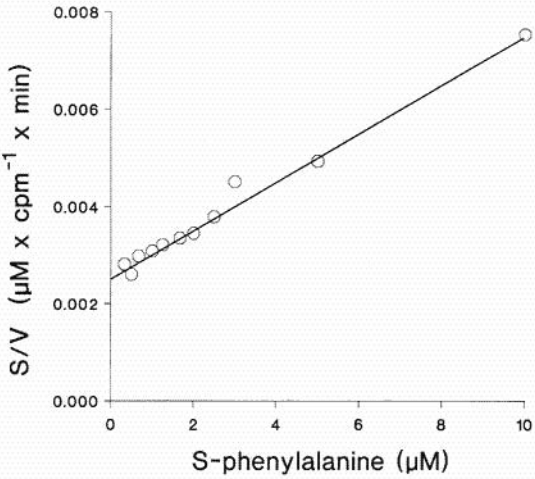


Fig. 1. Hanes plot (Bisswanger, 1979) of wheat germ *(Triticum aestivum)* PRS activity as a function of the (*S*)-phenylalanine concentration in the $tRNA^{phe}$-aminoacylation assay.

The apparent K_m values for $tRNA^{phe}$ (Table II) were measured with partially purified homologous $tRNA^{phe}$. The purified wheat germ tRNA-sample contained 10.6% $tRNA^{phe}$. Soybean PRS is able to aminoacylate $tRNA^{phe}$ from wheat germ in the heterologous system, the apparent K_m value (0.92 µM) being, however, ten times higher than in the homologous system (data not shown).

In the $tRNA^{phe}$-aminoacylation assay for PRS from baker's yeast we observed non-linear curves in the Hanes plots for both (*S*)-phenylalanine and homologous $tRNA^{phe}$, as well as for ATP (data

Table II. K_m values of the PRS from wheat germ *(Triticum aestivum)*, soybean cotyledons *(Glycine max)*, and baker's yeast *(Saccharomyces cerevisiae)*. Apparent K_m values for the different substrates in the $tRNA^{phe}$-aminoacylation and the ATP-PP_i exchange assays were determined for the enzymes from the three sources at pH 8.2, 7.8, and 7.4, respectively.

K_m values	Enzyme source: Wheat germ [µM]	Soybean [µM]	Yeast [µM]
$tRNA^{phe}$-aminoacylation assay			
(*S*)-Phenylalanine	5.2	0.9	5.5
$tRNA^{phe}$	0.34	0.09	0.09
ATP	347	31	360
ATP-PP_i exchange assay			
(*S*)-Phenylalanine	51	n.d.	65

not shown). (*S*)-Phenylalanine concentrations up to 8 μM and ATP concentrations between 0.1 to 2 mM were used to calculate the apparent K_m values (Table II). Substrate inhibition for homologous $tRNA^{phe}$ in concentrations above 1 μM was observed with the yeast enzyme.

Studies on the inhibition of the $tRNA^{phe}$-aminoacylation reaction

Inhibition studies were carried out by adding phenylalanine analogues (Table III) in four different concentrations up to 1 mM to the $tRNA^{phe}$-aminoacylation assay with 0.3, 0.5, 1.0, and 2.0 μM (*S*)-phenylalanine. Apparent K_i values were calculated, and the type of the inhibition was determined from Lineweaver-Burk plots (using the statistical procedures pointed out above) as well as from Dixon plots (Bisswanger, 1979).

As shown in Fig. 2, the phosphonic acid analogue of (*S*)-phenylalanine, (*R*)-APEP, inhibited the $tRNA^{phe}$-aminoacylation catalyzed by wheat germ PRS competitively with respect to (*S*)-phenylalanine, with an apparent K_i of 144 μM. The $tRNA^{phe}$-aminoacylation reactions catalyzed by the PRS from soybean and baker's yeast were also inhibited competitively by (*R*)-APEP, but inhibition of the yeast enzyme was weaker (Table III).

Ring-substituted derivatives of APEP were also tested in the $tRNA^{phe}$-aminoacylation assay. All of them had a lesser affinity to the three PRS than APEP itself (Table III); some were hardly inhibitory at all (apparent K_i values in the range of ≥ 1000 to $\gg 1000$ μM).

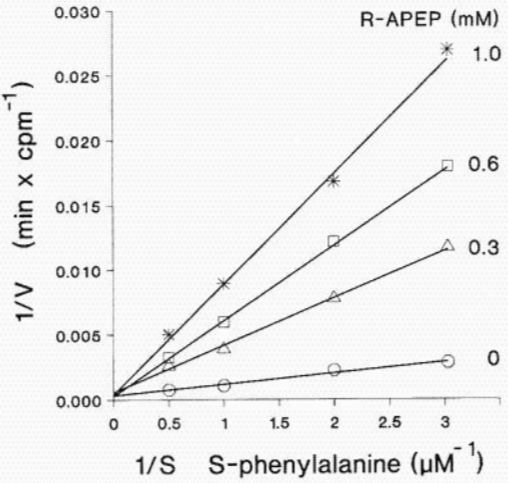


Fig. 2. Lineweaver-Burk plot (Bisswanger, 1979) for the inhibition of the wheat germ *(Triticum aestivum)* PRS by (*R*)-APEP in the $tRNA^{phe}$-aminoacylation assay.

Interestingly, (*R*)-*p*-fluoro-APEP and (*R,S*)-*p*-fluoro-APEP were non-competitive inhibitors of the $tRNA^{phe}$-aminoacylation catalyzed by wheat germ PRS (Table III). Inhibition of the yeast and the soybean enzymes by (*R*)- and (*R,S*)-*p*-fluoro-APEP was much weaker, and inhibition constants and the type of inhibition were therefore not determined (Table III).

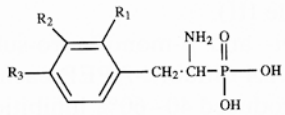
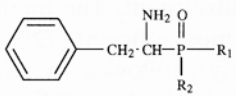
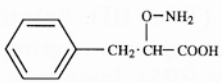
Racemic *o*-, *m*- and *p*-monofluoro-substituted APEP, as well as 2,4-difluoro-APEP, each at 1 mM concentration, produced 40–60% inhibition of the PRS from both wheat germ and soybean in the $tRNA^{phe}$-aminoacylation assay with 0.3 μM (*S*)-phenylalanine. There were no significant differences related to the position of the substituent.

However, using the same concentrations, ring-methylated (*R,S*)-APEP inhibited the PRS from wheat germ and soybean as a function of the position of the methyl substituent. The methyl group in *meta*-position was most efficient (72% and 35% inhibition, respectively), followed by *para*- (20% both) and *ortho*- (12% both). (*R,S*)-*m*-Methyl-APEP was a competitive inhibitor of soybean PRS with a K_i of 690 μM (Table III).

We found the phosphonous acid analogue of (*S*)-phenylalanine, (*R*)-APEPi, to be a competitive inhibitor of the $tRNA^{phe}$-aminoacylation of all three PRS we tested (Table III). Substitution of the hydrogen of the phosphonous group reduces the affinity for all three PRSs. Inhibition by (*R,S*)-APE-methyl-Pi was competitive for the PRS from baker's yeast. Inhibition decreased further when both the hydrogen and the hydroxyl of the phosphonous group were replaced by methyl groups (Table III). Racemic APEP, APEPi, APE-methyl-Pi, and APE-dimethyl-Pi, each at 1 mM concentration, inhibited the $tRNA^{phe}$-aminoacylation catalyzed by the wheat germ PRS by 78, 45, 21, and 14% respectively (phenylalanine concentration: 0.2 μM).

(*S*)-α-Hydrazino-β-phenylpropionic acid was found to be an uncompetitive inhibitor of baker's yeast PRS, with an apparent K_i of 543 μM. In contrast, this compound proved to be a competitive inhibitor of the soybean enzyme and a weak inhibitor of the wheat germ $tRNA^{phe}$-aminoacy-

Table III. Apparent K_i values of phenylalanine analogues in the $tRNA^{phe}$-aminoacylation reaction catalyzed by the PRS from wheat germ *(Triticum aestivum)*, soybean cotyledons *(Glycine max)*, and baker's yeast *(Saccharomyces cerevisiae)*, in comparison with the corresponding values for PAL from buckwheat *(Fagopyrum esculentum)*. The inhibition was competitive for all analogues for which apparent K_i values are given, with the exceptions indicated in the footnotes.

Enzyme source pH of assay		PAL[e] Buckwheat 8.8	PRS Wheat germ 8.2	PRS Soybean 7.8	PRS Yeast 7.4
K_m (*S*)-phenylalanine [μM]		45	5.2	0.9	5.5
Analogue			apparent K_i [μM]		
					
(*R*)-APEP	R_1 = H, R_2 = H, R_3 = H	1.5	144	130	1096
(*R,S*)-APEP	R_1 = H, R_2 = H, R_3 = H	2.6	221	340	≫1000
(*R,S*)-*o*-Methyl-APEP	R_1 = CH_3, R_2 = H, R_3 = H	12.3	[d]	[d]	[d]
(*R,S*)-*m*-Methyl-APEP	R_1 = H, R_2 = CH_3, R_3 = H	21.0	[d]	690	[d]
(*R,S*)-*p*-Methyl-APEP	R_1 = H, R_2 = H, R_3 = CH_3	780	[d]	[d]	[d]
(*R,S*)-*p*-Fluoro-APEP	R_1 = H, R_2 = H, R_3 = F	3.5	1051[b]	[d]	[d]
(*R*)-*p*-Fluoro-APEP	R_1 = H, R_2 = H, R_3 = F	2.8	300[a]	[d]	n.d.
					
(*R*)-APEPi	R_1 = H, R_2 = OH	35	315	160	117
(*R,S*)-APEPi	R_1 = H, R_2 = OH	110	741	300	134
(*R,S*)-APE-methyl-Pi	R_1 = CH_3, R_2 = OH	850	≥1000	[d]	230
(*R,S*)-APE-dimethyl-Pi	R_1 = CH_3, R_2 = CH_3	≫1000	≫1000	[d]	[d]
					
(*S*)-AOPP		0.0014	≫1000	≫1000	≫1000
(*R*)-AOPP		0.025	≫1000	≫1000	≫1000
(*S*)-α-Hydrazino-β-phenylpropionic acid		0.15	[d]	≥600	(543)[c]
(*R,S*)-α-Hydroxymethyl-phenylalanine		n.d.	1990	n.d.	M[d]
(*R,S*)-α-Difluoromethyl-phenylalanine		n.d.	[d]	1100	n.d.

[a] Non-competitive inhibition K_{ii} = 375 μM.
[b] Non-competitive inhibition K_{ii} = 924 μM.
[c] Uncompetitive inhibition K_{ii} = 543 μM.
[d] Weak inhibition, K_i ≥ 1000 μM to ≫ 1000 μM.
[e] Competitive inhibition, apparent K_i values from Amrhein (1986) and unpublished results of B. Laber and N. Amrhein.

Chemical structures of (1-amino-2-phenylethyl)phosphonic acid (APEP), (1-amino-2-phenylethyl)phosphonous acid (APEPi), (1-amino-2-phenylethyl)-methyl phosphonous acid (APE-methyl-Pi), and α-aminooxy-β-phenylpropionic acid (AOPP).

Other derivatives of (*R,S*)-APEP (*m*-trifluoromethyl-APEP, *o*-fluoro-APEP, *m*-fluoro-APEP, *p*-chloro-APEP, *p*-bromo-APEP, *p*-iodo-APEP, *p*-hydroxy-APEP, 2,4-dichloro-APEP, 3,4-dichloro-APEP, 3,4-dihydroxy-APEP, 2,4-difluoro-APEP, 2,6-diiodo-APEP, *p*-*tert*-butyl-APEP) were all found to have a lower affinity to the listed enzymes than the parent compound.

lation reaction, but because of its weak effect inhibition constants and type of inhibition were not determined (Table III). The aminooxy analogue of phenylalanine, AOPP, does not inhibit the $tRNA^{phe}$-aminoacylation reaction, irrespective of the source of the PRS (Table III).

Inhibition studies on the ATP-pyrophosphate exchange reaction

ATP-PP_i exchange assays were carried out using 2 mM concentrations of both (*S*)-phenylalanine and the respective phenylalanine analogue. The inhibition of the reaction by the analogue was compared to a control without analogue (0% inhibition) and a control without both, phenylalanine analogue and (*S*)-phenylalanine (100% inhibition). The ATP-PP_i exchange reaction catalyzed by wheat germ PRS was inhibited by (*R*)-APEP: 78%, (*R,S*)-APEP: 37%, (*R*)-APEPi: 53%, (*R,S*)-APEPi: 38%, (*R,S*)-APE-methyl-Pi: 11%, (*R,S*)-3,4-dichloro-APEP: 22%, and (*R,S*)-α-hydroxymethylphenylalanine: 7%. The yeast enzyme was also inhibited by (*R*)-APEP, (*R,S*)-APEP, (*R*)-APEPi, (*R,S*)-APEPi, and (*R,S*)-APE-methyl-Pi to comparable degrees. Other phenylalanine analogues and soybean PRS were not tested in the ATP-PP_i exchange reaction.

Studies on the activation of phenylalanine analogues

According to Biryukov *et al.* (1978), the α-aminophosphonous acid analogues of (*S*)-valine and (*S*)-methionine are substrates in the ATP-PP_i exchange reaction of (*S*)-valine- and (*S*)-methionine-tRNA synthetases from *E. coli*. The possible activation of the phenylalanine analogues (each at 2 mM concentration), was tested in the ATP-PP_i exchange assay for the PRS from wheat germ and yeast and compared with the activation of (*S*)-phenylalanine. No activation of these or any other phenylalanine analogue compiled in Table III was found. The activation of (*S*)-α-hydrazino-β-phenylpropionic acid previously reported for mungbean PRS (Norris *et al.*, 1975) could not be confirmed for the PRS from wheat germ and yeast.

Discussion

Most PRSs are tetramers with an $\alpha_2\beta_2$ subunit structure, as shown, for example, for the cytoplasmic PRSs of yeast and wheat germ (Joachimiak and Barciszewski, 1980; Carias and Julien, 1976). We confirmed here the results of Joachimiak and Barciszewski (1980) who found apparent molecular masses of 61 and 69 kDa for the two subunits of wheat germ PRS. In contrast, Carias and Julien (1976) determined molecular masses of 50 and 80 kDa. As an exception among the PRS, cytoplasmic PRS from soybean is a monomer of 80 kDa (Swamy and Pillay, 1980), a molecular mass also found among those of the proteins in our enzyme preparation.

The apparent K_m values determined for (*S*)-phenylalanine for wheat germ PRS (Table II) compare favourably with those determined by Carias and Julien (1976), *i.e.* 6.6 μM in the $tRNA^{phe}$-aminoacylation and 51 μM in the ATP-PP_i exchange reactions, and the apparent K_m value for $tRNA^{phe}$ from wheat germ is, likewise, within the same order of magnitude (0.34 μM *vs.* 2.7 μM). For the $tRNA^{phe}$-aminoacylation catalyzed by the soybean PRS we confirmed the apparent K_m values measured by Swamy and Pillay (1980), *i.e.* 0.45 μM for (*S*)-phenylalanine, 30 μM for ATP, and 0.08 μM for homologous $tRNA^{phe}$. For the PRS from baker's yeast, Hanes plots (Bisswanger, 1979) yielded non-linear curves for all substrates, as described by other authors (Fasiolo *et al.*, 1977; Paillez and Waller, 1984).

As concluded from previous inhibition studies (Lea and Norris, 1977; Lavrik and Moor, 1985; Loftfield, 1972), plants PRS is less specific for its amino acid substrate (phenylalanine) than other plant aminoacyl-tRNA synthetases, or even than PRS from other organisms. For the binding of (*S*)-phenylalanine to PRS only the benzene ring and the α-amino group are important, but not the carboxylic group. The positively charged α-amino group of the protonated form of (*S*)-phenylalanine is thought to bind to a negatively charged group in the active site of the enzyme molecule (Lea and Norris, 1977; Lavrik and Moor, 1985; Loftfield, 1972).

Our inhibition experiments support these general rules. For the PRSs from wheat germ, soybean, and baker's yeast, strong basicity of the

free α-amino group of the respective phenylalanine analogue is essential for its affinity to the enzyme.

The α-aminooxy group of AOPP, which is a strong competitive inhibitor of PAL from buckwheat (Table III), is less basic than the amino group of (*S*)-phenylalanine. In agreement with the above rule, we found that AOPP does not inhibit the $tRNA^{phe}$-aminoacylation catalyzed by all three PRS from the different sources (Table III). Likewise, AOPP does not inhibit the ATP-PP_i exchange reaction catalyzed by the PRS from wheat germ and baker's yeast, nor is it a substrate in this reaction. This result confirms the report of Norris *et al.* (1975) for the PRS from *Phaseolus aureus* that AOPP has no affinity to the enzyme.

In contrast to the above rule for PRS inhibitors, Amrhein (1986) concluded from inhibition studies with buckwheat PAL that the negatively charged carboxyl group of phenylalanine or of its analogues is important for their binding to the active site of PAL. With PAL, the lower basicity of the α-aminooxy group of AOPP appears to have a positive, rather than a negative effect on the affinity of these analogues to the enzyme (II), but steric factors as well as the chemical reactivity of the α-substituent must also be taken into consideration.

APEP was shown here to be a competitive inhibitor of the PRS from several organisms (Table IV), and racemic APEP has been reported to be a strong competitive inhibitor of the PRSs from horse chestnut *(Aesculus hippocastanum)* and from *A. parviflora* in the (*S*)-phenylalanine-activation reaction (Anderson and Fowden, 1970), (Table IV). (*R,S*)-APEP also inhibited the PRS from *E. coli* competitively both in the (*S*)-phenylalanine-activation reaction and in the $tRNA^{phe}$-aminoacylation reaction (Santi *et al.*, 1971) (Table IV), and we have shown here that APEP is a competitive inhibitor of the $tRNA^{phe}$-aminoacylation reaction of the PRS from wheat germ, soybean and yeast (Fig. 2, Tables III and IV).

When comparing the apparent inhibition constants of APEP for various PRSs (Table IV), we have to consider the fraction of APEP with a positively charged α-amino group at the pH of the respective assay mixture. Baylis *et al.* (1984) measured a pK of 10.5 for the α-amino group of the phosphonic acid analogue of valine. The pK of the α-amino group of phenylalanine (9.2) is only slightly lower than that of valine (9.4). If we assume a pK of approximately 10.3 of the α-amino group of APEP, at least 97% of the analogue are present in the positively charged form, at all pH values used in the assays for the PRSs (Table IV). Nonetheless, as can be seen from the K_i/K_m ratios in the $tRNA^{phe}$-aminoacylation assay (Table IV), there are large differences in the analogue's affinities towards PRS from different sources. The baker's yeast enzyme is inhibited to a lesser degree by APEP than the enzymes from wheat germ and soybean, which might be explained by the relatively low specificity for phenylalanine of PRS from plant sources (Lavrik and Moor, 1985; Lea and Norris, 1977).

The K_i/K_m ratio of buckwheat PAL for (*R*)-APEP is 0.03 (Table III). This demonstrates a much higher affinity of the analogue to PAL than

Table IV. APEP as a competitive inhibitor of the PRS from different organisms in the $tRNA^{phe}$-aminoacylation and the ATP-PP_i exchange assays.

Source of PRS	APEP	ATP-PP_i exchange			$tRNA^{phe}$-aminoacylation			Ref.
		K_m (*S*)-phe [μM]	K_i APEP [μM]	K_i/K_m	K_m (*S*)-phe [μM]	K_i APEP	K_i/K_m	
E. coli	*R,S*	50	40	0.8	5.5	180 μM	33	[a]
Baker's yeast	*R,S*	65	n.d.		5.5	⩾1 mM	⩾200	[c]
Baker's yeast	*R*	65	n.d.		5.5	1096 μM	199	[c]
A. hippocastanum	*R,S*	31	17	0.6	n.d.	n.d.		[b]
Wheat germ	*R,S*	51	n.d.		5.2	221 μM	43	[c]
Wheat germ	*R*	51	n.d.		5.2	144 μM	28	[c]
Soybean	*R,S*	n.d.	n.d.		0.9	340 μM	378	[c]
Soybean	*R*	n.d.	n.d.		0.9	130 μM	144	[c]

[a] Santi *et al.* (1971).
[b] Anderson and Fowden (1970a).
[c] This study.

to the different PRS (Table IV). The additional negative charge carried by the phosphonic acid group of (*R*)-APEP at the pH of the PAL assay is presumably responsible for the analogue's higher affinity for PAL in comparison to (*S*)-phenylalanine, the corresponding carboxylic acid, because the active site of PAL is thought to contain a counterion to the carboxylate group of phenylalanine (Amrhein, 1986). In contrast, while the carboxyl function of (*S*)-phenylalanine may be less important for its binding to the PRS, its substitution by the phosphonic acid group reduces the basicity of the essential α-amino group, leading to a lower affinity of PRS for APEP as compared to (*S*)-phenylalanine. All tested substitutions of APEP at the benzene ring result in compounds with weaker affinity to PRS than APEP itself (Table III).

APEPi is a competitive inhibitor of the $tRNA^{phe}$-aminoacylation reaction of the PRS from wheat germ, soybean and yeast (Table III). Comparing apparent inhibition constants for APEPi for the PRS of different origin, we again have to consider the fraction of APEPi carrying the essential positive charge on the α-amino group under the conditions of the assay. Baylis *et al.* (1984) measured a pK of 7.8 for the α-amino group of the phosphonous acid analogue of valine. If in analogy to the considerations for APEP we assume a pK of 7.6 for the α-amino group of APEPi, the following fractions will prevail in the positively charged form (at the pH values of the respective enzyme assay systems) (Table III): 61.3% (pH 7.4, PRS yeast), 38.7% (pH 7.8, PRS soybean), 20.1% (pH 8.2, PRS wheat germ), and 5.9% (pH 8.8, PAL buckwheat). In this case, the observed stronger inhibition of yeast PRS as compared to wheat germ PRS might also be explained by the 3-fold higher concentration of positively charged APEPi at the lower pH value of the yeast enzyme assay.

Because only at the pH of the assay for yeast PRS the other tested phenylalanine analogues were predominantly in the positively charged form, the K_i values of the compounds with different substitutions of the carboxylic group can only be compared for yeast. Substitution of the carboxylic group for the larger tetrahedral and doubly negatively charged phosphonic group results in a lower affinity to the yeast PRS. The phosphonous and methylphosphonous acid analogues with a single negative charge as the carboxylate group bind quite well to yeast PRS, whereas the dimethylphosphonous analogue has a low affinity for the PRS, either because of the missing negative charge or for steric reasons. APEPi and APEP also inhibited the ATP-PP_i exchange reaction catalyzed by wheat germ PRS. In contrast to the results of Biryukov *et al.* (1978), for the (*S*)-valine- and (*S*)-methionine-tRNA synthetases from *E. coli,* neither APEPi nor APEP were activated as substrates in the ATP-PP_i exchange reaction catalyzed by any of the PRS tested here.

A comparison of the data for APEP in Table IV reveals that the $tRNA^{phe}$-aminoacylation assay is more specific for (*S*)-phenylalanine than the ATP-PP_i exchange assay. Because of this, the $tRNA^{phe}$-aminoacylation assay is assumed to more accurately reflect the *in vivo* reaction of the PRS. The results obtained in this assay will allow a more reliable prediction of an *in vivo* inhibition of protein synthesis by a phenylalanine analogue. All three PRSs listed in Table III were less sensitive to inhibition by APEP in the $tRNA^{phe}$-aminoacylation assay by a factor of at least 800 as compared to buckwheat PAL. Therefore we may assume that inhibition of protein synthesis by potent inhibitors of PAL, such as APEP and AOPP, is not involved in their *in vivo* action. This assumption is corroborated by the observation that seeds germinate, and seedlings grow in the presence of these inhibitors (Laber *et al.,* 1986; Holländer *et al.* (1979). Lastly, while not tested in this study, the conformationally restricted phenylalanine analogue 2-aminoindan-2-phosphonic (AIP), a recently described powerful slow-binding inhibitor of PAL (Zoń and Amrhein, 1992), was reported by others not to inhibit PRS (Biryukov, personal communication, cited by Zoń and Amrhein, 1992). This confirms the rule established in this work that powerful PAL inhibitors are no, or at most only weak inhibitors of PRS.

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