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Kinetic analysis of the inhibition of phenylalanine ammonia-lyase by 2-aminoindan-2-phosphonic acid and other phenylalanine analogues

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Dedicated to Professor Dr. Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

The conformationally restricted phenylalanine analogue 2-aminoindan-2-phosphonic acid (AIP) inhibits phenylalanine ammonia-lyase (PAL) competitively in a time-dependent manner. This phenomenon was investigated in more detail with the heterologously expressed, highly purified homotetrameric PAL-1 isozyme from parsley. The kinetic analysis revealed that the enzyme-inhibitor complex is formed in a single "slow" step with an association rate of $k_2 = 2.6 \pm 0.04\ 10^4\ M^{-1}\ s^{-1}$. The inhibition is reversible with a dissociation rate of $k_{-2} = 1.8 \pm 0.04\ 10^{-4}\ s^{-1}$ and an equilibrium constant of $K_i = 7 \pm 2$ nM. The previously described PAL inhibitor *(S)*-2-aminooxy-3-phenylpropanoic acid [*(S)*-AOPP] was also found to be a slow-binding inhibitor of PAL-1. The carboxyl analogue of AIP, 2-aminoindan-2-carboxylic acid, served as a substrate of PAL-1 and was converted to indene-2-carboxylic acid.

Keywords: *Petroselinum crispum*; 2-Aminoindan-2-phosphonic acid (AIP); Phenylalanine ammonia-lyase (PAL); Slow-binding enzyme inhibitor

1. Introduction

Inhibitors of enzymes are valuable tools in biochemical and physiological studies and may serve, depending on the organism which harbours the target enzyme, as drugs, toxins, antibiotics, herbicides etc. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the elimination of ammonia from *(S)*-phenylalanine to yield *(E)*-cinnamic acid. In higher plants, a substantial portion of the fixed carbon is channeled by PAL into the phenylpropanoid pathway (Hanson and Havir, 1981). *(E)*-Cinnamic acid is the precursor of a broad spectrum of compounds, such as flavonoids, coumarins, lignins, phenolic compounds, alkaloids etc. Because of the key function of PAL at a branching point of metabolism, inhibitors of this enzyme are in use to study the physiological role of compounds which are biosynthetically derived from *(E)*-cinnamic acid (Amrhein, 1986). Since *(S)*-phenylalanine is a substrate in diverse reactions in metabolism, including the biosynthesis of polypeptides, it is necessary to have inhibitors available which are strictly specific for the desired reaction. Non-specific inhibitors will interfere with several biosynthetic pathways and this will therefore complicate or render impossible the interpretation of the effects of an inhibitor observed in vivo.

The design of inhibitors of PAL and the analysis of their effects on the isolated enzyme as well as on whole plants has a long tradition in our groups (Janas et al., 1985; Amrhein, 1986). 2-Aminooxyacetate (AOA), a known transaminase inhibitor, with modest inhibitory action on PAL, was first used for such studies (Amrhein et al., 1976). Then the *O*-hydroxylamine analogue of

Abbreviations: AIC, 2-aminoindan-2-carboxylic acid; AIP, 2-aminoindan-2-phosphonic acid; AMPP, 2-aminomethyl-3-phenylpropanoic acid; AOPP, 2-aminooxy-3-phenylpropanoic acid; APEP, 1-amino-2-phenylethylphosphonic acid; E, enzyme concentration; E_t, total enzyme concentration; HAL, histidine ammonia-lyase; I, inhibitor concentration; I_t, total inhibitor concentration; k_x, first- or second-order rate constants, positive or negative number subscripts are assigned for the forward and backward rate constants respectively.; PAL, phenylalanine ammonia-lyase; PAL-1, phenylalanine ammonia-lyase isoenzyme 1 from *Petroselinum crispum*; v, v_s, v_o, enzyme reaction velocities measured at time t, steady-state and time zero; v_i, enzyme reaction velocity measured in the presence of inhibitor at steady-state.

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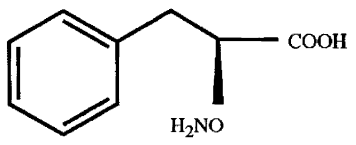
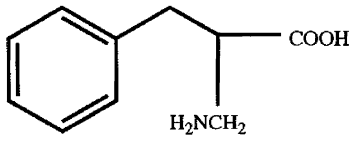
(S)-phenylalanine, *(S)*-2-aminooxy-3-phenylpropanoic acid (*(S)*-AOPP) (Amrhein and Gödeke, 1977), which was by several orders of magnitude superior to AOA as a PAL inhibitor, was introduced. *(S)*-AOPP inhibited PAL from *Fagopyrum esculentum* Moench (buckwheat) and the yeast *Rhodotorula glutinis* with K_i/K_m ratios of 0.00003 and 0.0002, respectively (Amrhein and Gödeke, 1977). From K_d values for PAL of *(S)*-phenylalanine and *(S)*-AOPP and other compounds, and from the observation that *(S)*-AOPP and *(R)*-AOPP have similar K_d values, Hanson (1981) concluded that *(S)*-AOPP is a reaction intermediate analogue of the reaction catalyzed by PAL. *(S)*-AOPP effectively inhibits the phenylpropanoid pathway in vivo (Amrhein and Holländer, 1979) and produces a selective 20-fold accumulation of *(S)*-phenylalanine in buckwheat hypocotyls (Holländer et al., 1979). Because of these advantages, *(S)*-AOPP has been used in many in vivo studies (see Amrhein, 1986). But considering its low K_i for PAL, *(S)*-AOPP is a surprisingly inefficient inhibitor in vivo and furthermore it cannot be considered an absolutely specific inhibitor of PAL (De-Eknamkul and Ellis, 1987). Further investigations revealed that *(R)*-1-amino-2-phenylethylphosphonic acid [*(R)*-APEP], the phosphonic analogue of *(S)*-phenylalanine, is a competitive inhibitor of PAL (Janas et al., 1985; Laber et al., 1986). Even though the K_i-value of *(R)*-APEP for buckwheat PAL is three orders of magnitude higher than that of *(S)*-AOPP, its efficiency in inhibiting the synthesis of phenylpropanoid compounds in vivo was quite comparable to that of *(S)*-AOPP (Laber et al., 1986). The promising results obtained with the phosphonic analogue *(R)*-APEP encouraged the synthesis and evaluation of other phosphonic analogues of phenylalanine. Among these, 2-aminoindan-2-phosphonic acid (AIP), the conformationally restricted cyclic analogue, was found to be a particularly effective inhibitor of PAL, both in vivo and in vitro (Zoń and Amrhein, 1992). While AIP has since been used in numerous studies to disrupt phenylpropanoid metabolism in vivo (see Zoń et al., 2002, for references), a detailed analysis of its interaction with PAL in vitro is missing.

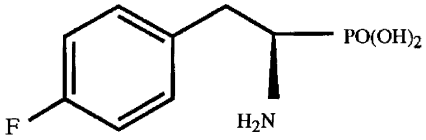
In the present work, we have analyzed in depth the inhibition of PAL by AIP and other phenylalanine analogues, the structures of which are given in Fig. 1. While the interaction of PAL and AIP had previously been investigated in crude extracts from illuminated buckwheat hypocotyls, the present investigation utilized a pure, clearly defined homotetrameric PAL preparation which was obtained by expressing the parsley PAL-1 isoenzyme in *Escherichia coli* (Appert et al., 1994).



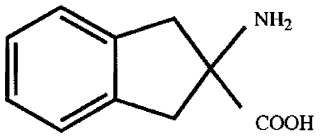
(S)-2-aminooxy-3-phenylpropanoic acid ((S)-AOPP)



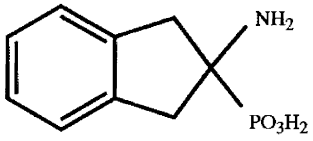
(±)-2-aminomethyl-3-phenylpropanoic acid (AMPP)



(R)-1-amino-2-(4-fluorophenyl)ethylphosphonic acid ((*R*)-4'-F-APEP)



2-aminoindan-2-carboxylic acid (AIC)



2-aminoindan-2-phosphonic acid (AIP)

Fig. 1. Structures of compounds tested for inhibition of PAL.

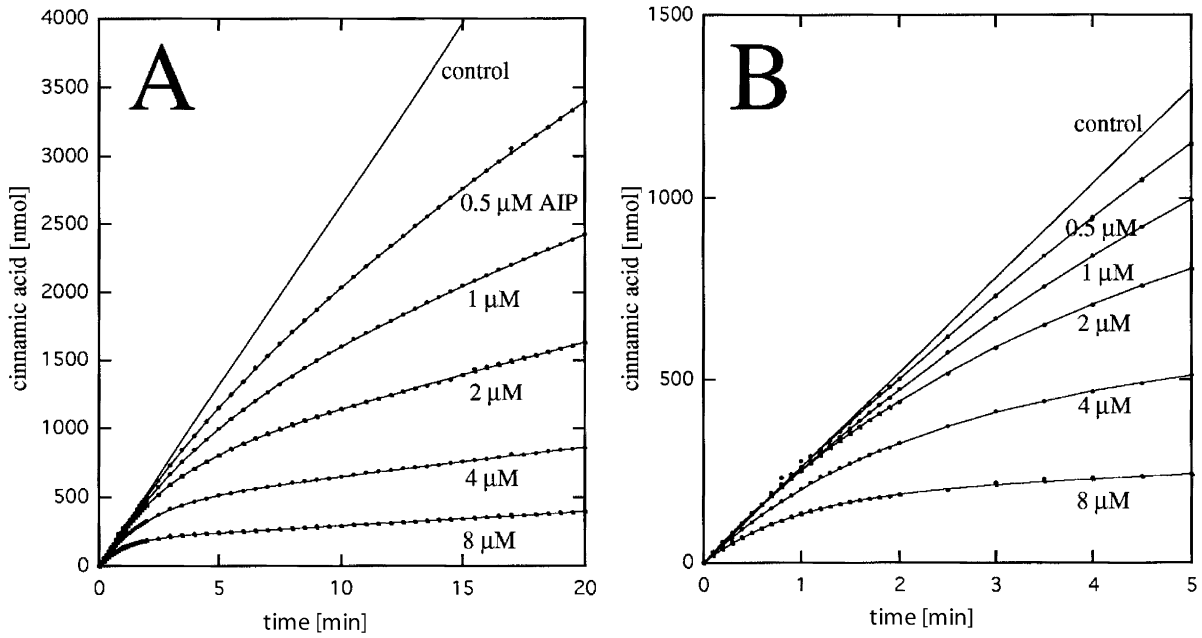


Fig. 2. Assay progress curves in the presence of AIP. PAL was incubated at 40 °C in 0.2 M borate/NaOH pH 8.8 containing 1 mM *(S)*-phenylalanine and increasing concentrations of AIP. The reaction was started by the addition of the enzyme. Points represent measured values and the curves were generated by fitting the data to Eq. (2). A: time scale 0–20 min, B: time scale 0–5 min.

2. Results

2.1. Inhibition of PAL by AIP

It had previously been shown that AIP is a competitive inhibitor of PAL in crude extracts from buckwheat hypocotyls (Zoń and Amrhein, 1992). Competitive inhibition by AIP was confirmed for the purified parsley enzyme, and a K_i of 25±4 nM was obtained from Dixon plots, when v at steady-state level was plotted against substrate concentrations in the presence of different inhibitor concentrations (data not shown). In contrast to classical competitive inhibitors which establish the steady-state level of inhibition very rapidly (<1 s), progress curves for the interaction of PAL with AIP revealed inhibition of the enzyme activity in a time-dependent manner (Fig. 2). The same steady-state level of remaining activity was obtained irrespective of whether the enzyme was preincubated with the inhibitor and the reaction was then started by addition of the substrate, or whether the reaction was started by addition of the enzyme to a mixture of substrate and inhibitor (Fig. 3). This behaviour indicated i) that AIP is a slow binding inhibitor, and ii) that the inhibition is reversible. The reversibility of the binding excludes that AIP is either a group specific reagent, a suicide substrate or an affinity label which all may produce similar inhibition patterns like that observed for AIP. When a steady-state level of inhibition had been achieved and the enzyme was then denatured by heat, addition of fresh, active enzyme led precisely to the same pattern of inhibition as in the previous incubation, excluding the possibility that the observed steady-state levels are due to the inactivation or consummation of AIP during the incubation with PAL (data not shown). Enzyme inhibitors with characteristics such as those observed for AIP have been termed slow-binding inhibitors (Morrison, 1982). Methods for the analysis of this phenomenon have been published by various authors (Cha, 1975; Morrison and Walsh, 1987; Schloss, 1989).

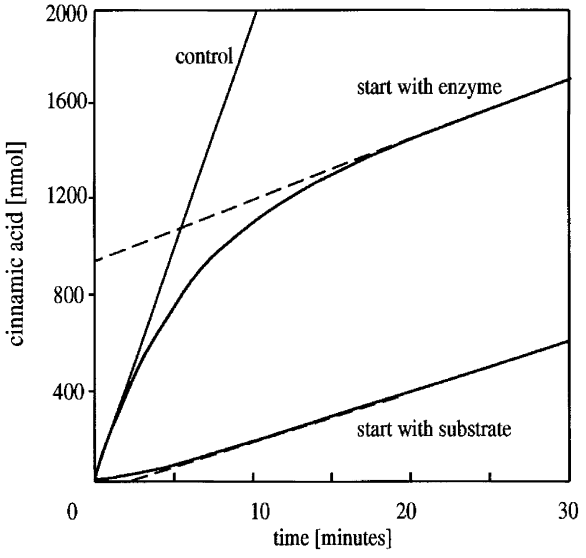
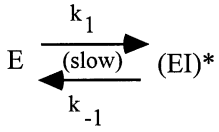


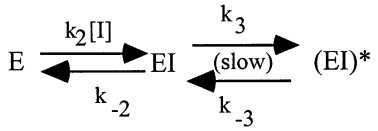
Fig. 3. Time-dependent arrival at steady-state of PAL inhibition by AIP. PAL was either incubated at 40 °C in 0.2 M borate/NaOH pH 8.8 in the presence of 1 µM AIP and then the reaction was started by the addition of substrate or the reaction was started by the addition of enzyme.

There are two simple mechanisms which can account for a slow onset of inhibition:

mechanism A



mechanism B



In mechanism A, the binding of the inhibitor to the enzyme is slow and directly produces the final enzyme–inhibitor complex. In mechanism B, an EI complex is rapidly formed which then undergoes a slow tightening or isomerisation to EI*. For mechanism A the initial reaction velocity (v_0) will be independent of the inhibitor concentration, whereas for mechanism B the formation of the inhibitory complex exhibits biphasic pseudo-first-order kinetics which results in a steady-state level of inhibition.

Progress curves, monitoring the product formation by the enzyme in the presence of different concentrations of inhibitor, revealed that the initial velocity did not vary with the inhibitor concentration (Fig. 2). According to Cha (1975, 1976a, b) the data from such progress curves which consist of an exponential and a linear phase, can be utilized to determine the rate constants for the formation of the (EI)* complex:

$$v(t) = v_s + (v_0 - v_s)e^{-kt} \quad (1)$$

whereby $v(t)$ is the observed reaction velocity at a given time, v_0 is the initial velocity, v_s the steady-state velocity of the reaction, and $k = k_{-1}(1 + I/K_i + S/K_m)/(1 + S/K_m)$. Eq. (1) is applicable independent of whether the reaction is started by the addition of the enzyme to the reaction mixture containing substrate and inhibitor, or by the addition of substrate after pre-incubation of the enzyme and inhibitor. Integration of Eq. (1) results in Eq. (2), which allows the determination of v_s, v_0 and k by fitting the data for the formed product (P) as a function of time.

$$P(t) = v_s t - (v_s - v_0)\left(1 - e^{-kt}\right)/k \quad (2)$$

whereby $k = k_{-1} + k_1 I/(1 + S/K_a)$ (Williams and Morrison, 1979). The v_o values at different AIP concentrations conform with mechanism A.

Alternatively, the interaction of AIP with PAL was allowed in the absence of the substrate *(S)*-phenylalanine. At intervals the reaction was started by addition of *(S)*-phenylalanine and the initial velocities were measured (Fig. 4). The rate at which the equilibrium is obtained in the absence of substrate is defined by Eq. (3):

$$\%\ \text{remaining activity} = (100\%)[B + (A - B)e^{-kt}] \quad (3)$$

whereby $A = 1$, $B = 1/(1 + I/K_i)$ and $k = k_{-1} + k_1 I$ (Schloss, 1989). The inhibition curves clearly orginated at 100% activity, again indicating that mechanism A is valid for the mode of interference of AIP with PAL.

The availability of *(n)*-^{3}H-AIP allowed to determine the K_d-value for AIP by equilibrium dialysis. A K_d-value of 8.2 ± 1.0 nM, which is in reasonably good agreement with the K_i-value determined (Table 1), indicates that AIP can not be considered a slow, *tight* binding inhibitor (Morrison and Walsh, 1987), because for effective inhibition of PAL under the normal experimental conditions, the AIP concentration [I_t] must be larger than [E_t] by two orders of magnitude.

2.2. Inhibition of PAL by other phenylalanine analogues

The availability of a purified homotetrameric PAL isoenzyme prompted us to reinvestigate the interaction with PAL of several inhibitors which previously had only been evaluated with PAL in crude plant extracts or

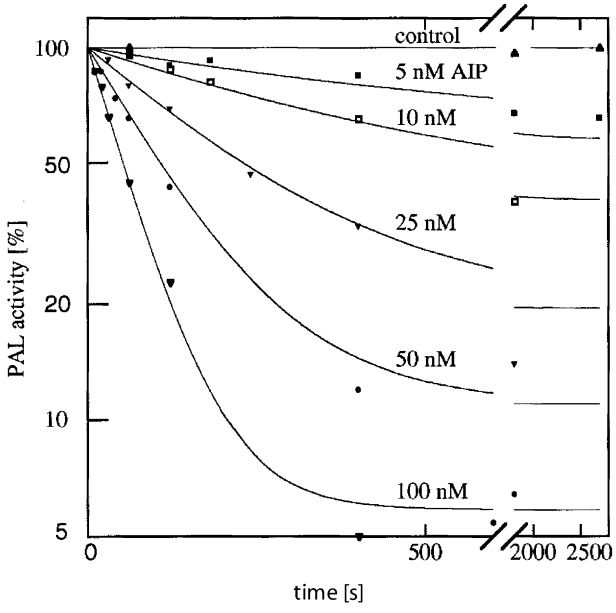


Fig. 4. Time-dependent inhibition of PAL by AIP. PAL was incubated at 40 °C in 900 µl 0.2 M borate/NaOH pH 8.8 containing increasing concentrations of AIP. PAL was added at $t = 0$. At intervals the enzymatic reaction was started by the addition of 100 µl 10 mM L-phenylalanine and the initial velocity was measured. Points represent measured values from the experiment and the curves were generated by fitting the data to Eq. (3). 100% activity = 650 pkat.

Table 1
Kinetic and inhibition constants of inhibitors[a]

Compound	K_i (μM)	k_2 10^4 (M^{-1} s^{-1})	k_{-2} 10^{-4} (s^{-1})	I_{50} (μM)
AIP(a)	0.025±0.004			1.5
AIP(b)	0.007±0.002	2.6±0.04	1.8±0.4	1.5
AIP(c)	0.007±0.0007	14.1±0.6	9.2±0.9	1.5
(S)-AOPP(b)	0.00038±0.00005	42.6±0.2	1.6±0.2	10
AMPP(a)[b]	8.9±0.4			2000
(R)-4′-F-APEP(a)	1.0±0.1			50
AIC(a)	1.9±0.1			3000

[a] K_i, k_2 and k_{-2} were determined by fitting the reaction velocities measured in the presence of inhibitor at steady-state to Eq. (4) which describes competitive inhibitors (a), by fitting data for product formed at time (t) from progression curves to Eq. (2) (b), or by measuring the remaining enzymatic activity after incubation of the enzyme and the inhibitor for a given time in absence of the substrate and fitting the results to Eq. (3) (c). In vivo I_{50} values were determined by measuring the inhibition of light induced anthocyanin synthesis in buckwheat hypocotyls (Amrhein et al., 1976).
[b] Racemic compound.

partially purified from yeast. *(S)*-AOPP (Amrhein and Gödeke, 1977) was confirmed to be a powerful competitive inhibitor of PAL but, in addition, our refined analysis revealed that it is also a slow binding inhibitor. The inhibitor and rate constants were determined using Eq. (2) (Table 1). On the other hand, AMPP (Zoń and Laber, 1988) and AIC (Zoń and Amrhein, 1992) are simple competitive inhibitors of PAL; their inhibition constants were determined by fitting the kinetic data to Eq. (4) (Schloss, 1989) (Table 1):

$$v_s = V_{max}S/(K_m(1 + I/K_i) + S) \quad (4)$$

Because *(R)*-APEP (Laber et al., 1986) was no longer available, we used *(R)*-4′-F-APEP, instead, which when tested with buckwheat PAL had inhibitory properties quite similar to those of APEP [K_i: *(R)*-APEP 1.5 μM; *(S)*-APEP 11.6 μM; *(R)*-4′-F-APEP 2.8 μM; *(S)*-4′-F-APEP 13.5 μM] (Laber and Amrhein, unpublished). Its inhibition of PAL activity was also of the simple competitive type (Table 1).

When AIC was incubated with the enzyme in the absence of *(S)*-phenylalanine, it became evident that this analogue is a substrate of PAL. HPLC of the respective incubation mixture at different time intervals, revealed a new UV-absorbing peak, which increased with incubation time. The product was isolated by HPLC and rechromatographed by GC. Authentic indene-2-carboxylic acid, the expected product of the reaction of PAL and AIC, co-eluted both on HPLC and on GC with the reaction product from AIC. The MS fragmentation pattern of the isolated reaction product and of authentic indene-2-carboxylic acid were identical (methyl ester of indene-2-carboxylic acid: m/z 174, 129, 115). The K_m value for AIC could not be determined because of the limitations of the spectrophotometric assay, but it must be below 1 μM. The catalytic-center activity for AIC was determined to be 0.5 s^{-1}.

3. Discussion

All results obtained in this study are consistent with the notion that AIP is a reversible competitive inhibitor of parsley PAL-1. However in contrast to classical inhibition by competitive inhibitors, the steady-state level of inhibition is established only slowly. Reaction intermediate analogues of enzymatic reactions often exhibit slow association and dissociation rates and are also extremely potent inhibitors (Morrison and Walsh, 1987; Schloss, 1988, 1989). The molecular explanation of slow binding is a reversible, time-dependent alteration of either the enzyme or of the inhibitor. For the enzyme, this "isomerization" can involve reorientation of the protein (conformational change), or of the water structure at the active site, or a change of the oligomeric state. For the inhibitor, the change can involve the hydration/dehydration of a carbonyl residue, a change in the ionization state, or a conformational change (Schloss, 1988). These isomerization reactions can take place either in solution (mechanism A) or in the enzyme-inhibitor complex (mechanism B). The inhibitory potency of reaction intermediate analogues has its origin in the principle of catalysis. To facilitate a reaction, the high energy reaction intermediate has to be stabilized. This stabilization decreases the energy required for the reaction intermediate and therefore the reaction proceeds at a faster rate. The stabilization is achieved by binding the substrate in the reaction intermediate state more tightly than in the ground state.

We have shown here that both *(S)*-AOPP and AIP are slow binding inhibitors of parsley PAL-1. From both energetic and steric observations *(S)*-AOPP had previously been proposed to be a reaction intermediate analogue of PAL (Hanson, 1981). If it is assumed that both inhibitors are reaction intermediate analogues, the cause of the slow binding would be a conformational change in the structure of PAL. The K_i values of the two inhibitors would then suggest that *(S)*-AOPP is the structure that resembles best the reaction intermediate. However, the arguments put forward by Hanson (1981) were based on a proposed mechanism for PAL in which the α-amino group of the substrate is initially added to the prosthetic group of PAL, at that time believed to be a dehydroalanine. Schuster and Rétey (1995) proposed a mechanism involving an electrophilic attack at the phenyl ring by the prosthetic group of PAL. Homology of PAL with histidine ammonia-lyase (HAL) whose structure has been solved by X-ray crystallography (Schwede et al., 1999), as well as recent direct spectroscopic evidence obtained for PAL (Röther et al., 2000), suggest that HAL and PAL share a novel prosthetic

group, i.e. 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO), which acts as the electrophile. Röther et al. (2002) then built a homology model of PAL based on the structure of HAL as well as on site-directed mutagenesis experiments, which supports a mechanism in which MIO attacks the phenyl ring of the substrate. Future efforts should therefore be directed towards fitting the slow-binding inhibitors into the catalytic site of the homology model of Röther et al. (2002). It should be noted here that maize PAL, which also accepts *(S)*-tyrosine as substrate (Rösler et al., 1997), has a considerably higher Km for *(S)*-phenylalanine and is far less sensitive to inhibition by AIP and *(S)*-AOPP than parsley PAL (Rösler and Amrhein, unpublished). With maize PAL, the two inhibitors do not display slow-binding characteristics. Ultimately, it will be interesting to compare the geometry of the active sites of the enzymes from these two different sources in order to explain the disparity in their catalytic powers.

AIC is an alternative substrate of PAL. It has an at least 20-fold lower K_m compared to that of *(S)*-phenylalanine (17.2 μM). The higher affinity of AIC may be explained by its reaction intermediate like structure. On the other hand, the turnover number of AIC is dramatically reduced compared to the natural substrate. This may be explained by the constraints of the product which has a delocalized flat π-system that has to build up into a fixed ring structure. Interestingly, AMPP just weakly inhibits PAL in a time-independent manner, suggesting that AMPP is not a reaction intermediate analogue. AMPP and *(S)*-AOPP are isosteric compounds, with the methylene group in AMPP residing at the position of the oxygen in *(S)*-AOPP. This replacement results in a slightly different position of the amino group relative to the plane of the benzene ring (C–O and O–N bonds are shorter than C–C and C–N bonds) but also drastically increases the basicity of the amino group. In addition, the oxygen in *(S)*-AOPP has two lone electron pairs available for interaction with PAL via hydrogen bonds. The hydrazine analogue of *(S)*-phenylalanine, which carries a single lone electron pair at the NH-group bridging the amino group with the 3-phenylpropanoic acid fragment, is also a strong inhibitor of PAL, albeit with a nearly 100-fold reduced potency as compared to *(S)*-AOPP (Amrhein, 1986).

The efficiency of a PAL inhibitor in vivo can be evaluated by measuring its effect on light induced anthocyanin synthesis [which requires *(E)*-cinnamic acid as precursor] in buckwheat (Amrhein et al., 1976) as well as by the selective accumulation of *(S)*-phenylalanine in the tissue upon inhibitor treatment. A 50% inhibition of anthocyanin synthesis was achieved with a concentration of AIP 100 times lower than those of *(S)*-AOPP and *(R)*-APEP required to produce the same inhibition. Furthermore, a concentration of 10 μM AIP was sufficient to produce a 20-fold accumulation of *(S)*-phenylalanine whereas for *(S)*-AOPP and *(R)*-APEP a concentration of 1 mM was required (Zoń and Amrhein, 1992). AIP is therefore presently the most potent in vivo inhibitor of PAL available and is thus a useful tool to study various aspects of aromatic metabolism in plants.

4. Experimental

4.1. Chemicals

The following compounds were synthesized according to published procedures: AIP and AIC (Zoń and Amrhein, 1992), AMPP (Zoń and Laber, 1988), *(S)*-AOPP (Laber et al., 1986); *(R)*-4′-F-APEP was provided by Dr. L. Maier (Ciba-Geigy, Basel, CH).

Indene-2-carboxylic acid is readily obtained by the procedure of Liebermann and Zsuffa (1911). In a sealed ampoule, indene (1.4 ml, 0.0120 mol) and oxalyl chloride (2.4 ml, 0.0279 mol) were heated at 140 °C for 8 h. Then, the reaction mixture was transferred into a flask and all volatile components were evaporated under reduced pressure. The residue was dissolved in a saturated solution of sodium bicarbonate (50 ml) and heated to boiling with charcoal. The filtrate was acidified with concentrated hydrochloric acid (3.6 ml) to pH ~1 and the crude product was precipitated. The crude acid (1.4 g) was purified by sublimation (120 °C/1 mm Hg) yielding pure indene-2-carboxylic acid: 0.70 g (36%); m.p. 250–252 °C (ref. as above 234 °C). IR (KBr): $\nu = 760, 1210, 1270, 1575, 1590, 1690\ cm^{-1}$. ^1H NMR (80 MHz, CD_3COCD_3, TMS): $\delta = 3.62$ ppm (*m*, 2H, CH_2) and 7.1–7.9 ppm (*m*, 5H, $H_{arom.}$ and CH = C). UV: $\lambda_{max} = 280$ nm $\varepsilon = 1.4 \times 10^7\ cm^2\ mol^{-1}$.

(n)-^{3}H-AIP (486 GBq/mol) was custom-prepared from delivered AIP by Amersham (Buckinghamshire, GB) by exchange in a tritium gas atmosphere. The specific activity of the product was determined by comparing the inhibition of PAL by the labeled compound with the inhibition by a series of defined concentrations of unlabeled AIP.

Recombinant parsley PAL-1 was obtained as described by Appert al. (1994).

4.2. Assay for PAL

PAL activity was routinely assayed by following *(E)*-cinnamic acid formation at 280 nm in a DU-70 spectrophotometer (Beckman, Zürich, CH) at 40 °C in 0.2 M borate/NaOH, pH 8.8, containing 1 mM *(S)*-phenylalanine.

4.3. Data analysis

The kinetic data were analyzed using a computer program for nonlinear regressions (Sigma Plot, Jandel Scientific, Corte Madera, CA, USA). Initial clues for the

values of the parameters necessary for fitting equations to the measured variables, were obtained by using graphical procedures described by Henderson (1972) and Cha (1975).

4.4. Stopped-flow kinetics

Rapid kinetics were performed using the rapid kinetics spectrometer accessory RX.1000 (Applied Photophysics, Leatherhead, UK).

4.5. Analysis of reaction products

After incubation with PAL, reaction product originating from 2-aminoindan-2-carboxylic acid (AIC) was separated on a C-18 reversed phase column (μBONDPAK™C18, Waters, Millipore, Volketswil, CH) using a linear gradient from 5% acetic acid to 20% acetic acid/40% acetonitrile in water. The HPLC-purified reaction product from 2-aminoindan-2-carboxylic acid (AIC) was separated both as the free acid and as its methylester by GC on a Finnigan MAT-Magnum instrument equipped with a 25 m column (ID = 0.25 mm, film thickness = 0.25 μm, DB17, J&W Scientific, UK) using a temperature gradient from 80 to 250 °C. The purified substances were analyzed by MS. The mass spectrometer was operated at 70 eV.

4.6. Determination of the catalytic-center activity

The catalytic-center activity of PAL for the alternative substrate AIC was determined by comparing the rate of product formation with that for *(S)*-phenylalanine. The previously determined catalytic-center activity for PAL (40 s^{-1} per active site, at 40 °C; Appert et al., 1994) was used to calculate the catalytic-center activity for the alternative substrate.

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