1-Aminocyclopropanephosphonate: Time-Dependent Inactivation of 1-Aminocyclopropanecarboxylate Deaminase and *Bacillus stearothermophilus* Alanine Racemase by Slow Dissociation Behavior†

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ABSTRACT: 1-Aminocyclopropanephosphonate (ACPP) was synthesized, and its effects on the pyridoxal 5′-phosphate linked enzymes 1-aminocyclopropanecarboxylate (ACPC) deaminase from *Pseudomonas* sp. ACPC and alanine racemase from *Bacillus stearothermophilus* were studied. ACPP was found to be a potent inhibitor of both enzymes with K_m/K_i ratios of 500 and 2000, respectively. Inhibition for both enzymes was characterized by slow-binding (second-order rate constants <150 M^{-1} s^{-1}) slow-dissociating behavior. Analysis of the pre-steady-state kinetics revealed a kinetically detectable intermediate E·I complex in the inhibition mechanism for the racemase but not for the deaminase. The one-step deaminase inhibition

$$E + ACPP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ACPP \qquad E = ACPC \text{ deaminase; } K_i = 5\ \mu M$$

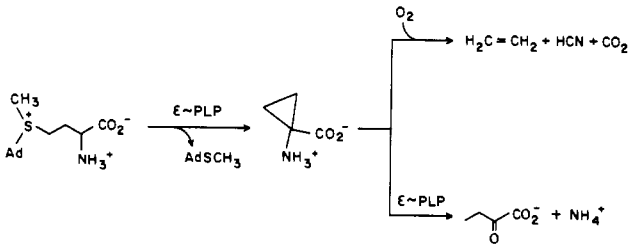
$$E + ACPP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ACPP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}ACPP^* \qquad E = \text{alanine racemase; } K_i = 8\ mM;\ K_i^* = 2\ \mu M$$

mechanism had an association rate constant (k_1) of 100 M^{-1} s^{-1}, a value 10^6-fold slower than diffusion, suggesting either a slow alignment of the inhibitor at the enzyme active site or, more likely, the same mechanism as followed by racemase but with an E·I to E·I* conversion rate (k_3) that is sufficiently fast on the steady-state time scale so as to hinder detection of the initial weakly associated E·I intermediate. The E to E·I transition for the deaminase was further monitored by ultraviolet–visible and circular dichroism (CD) spectroscopies and found to exhibit a time-dependent shift in the visible absorption spectrum λ_{max} from 418 nm for the native enzyme to 333 nm at steady state, again consistent with a rapid E to E·I and slow E·I to E·I* behavior. A rate constant for the absorbance shift of 150 M^{-1} s^{-1} was consistent with the k_1 calculated in the inhibition studies. The CD spectra showed a similar absorbance shift along with a change in the optical rotation direction. Sodium borohydride failed to reduce the deaminase–ACPP complex, suggesting that the 333-nm absorbance might arise from formation of a stable hemiaminal.

The cyclopropyl amino acid 1-aminocyclopropane-1-carboxylate (ACPC)[1] exists in trace amounts in the tissues of many plants (Yang & Hoffman, 1984). Plants synthesize ACPC from methionine by using ACPC synthase, an enzyme that catalyzes the formation of the cyclopropyl ring by an internal cyclization between carbons C-2 and C-4 of *S*-adenosylmethionine (Boller et al., 1979). ACPC is catabolized in nature by at least two different pathways. In plants, ACPC is converted by membrane-bound enzymes to the fruit-ripening wound-healing hormone ethylene (Adams & Yang, 1979; Lussen et al., 1979; Konze & Kende, 1979). The chemistry of this oxygen-dependent cyclopropane fragmentation has been investigated in several model studies and appears to follow a free-radical mechanism initiated by a one-electron oxidation of the ACPC amino group (Pirrung, 1983; Adlington et al. 1982, 1983; Peiser et al., 1984). The second ACPC-metabolizing pathway is present in some bacteria, yeast, and fungi and utilizes the ring-cleaving enzyme ACPC deaminase to convert ACPC to α-ketobutyrate and ammonia (Honma & Shimonura, 1978). (See Scheme I.)

ACPC synthase and ACPC deaminase are both PLP-linked enzymes and are the only coenzyme B_6 dependent enzymes known to make or break cyclopropane rings (Liu & Walsh, 1987). The synthase carries out a formal α-anion attack on the γ-carbon of the methionyl portion of *S*-adenosylmethionine with concurrent expulsion of 5-(thiomethyl)adenosine

Scheme I: ACPC Metabolic Pathways



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[1] Abbreviations: ACPC, 1-aminocyclopropanecarboxylate; PLP, pyridoxal 5′-phosphate; Ala-P, (1-aminoethyl)phosphonate; ACPP, 1-aminocyclopropanephosphonate; LDH, L-lactate dehydrogenase; D-AAO, D-amino acid oxidase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CD, circular dichroism; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; m, multiplet; TFA, trifluoroacetic acid; TEBA, triethylbenzylammonium chloride; TEA, triethylamine; TSP, sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate; E·I, enzyme–inhibitor initial complex; E·I*, enzyme–inhibitor complex after isomerization; k_{obsd}, rate of approach to steady-state equilibrium; k_{rgn}, rate of regain in enzymatic activity upon dilution of fully inhibited enzyme; PMP, pyridoxamine phosphate.

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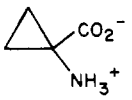
(Wiesendanger et al., 1986a,b). The deaminase, on the other hand, must effect a Cα–Cβ bond cleavage without obvious means of generating a Cα-carbanion or having available a retro aldol mechanism of the type utilized by serine transhydroxymethylase (Walsh, 1979). We have reported elsewhere (Walsh et al., 1981; Hill et al., 1984; Liu et al., 1984) a number of mechanistic and stereochemical probes aimed at delineating the reaction course of this unusual ring-cleaving reaction.

During our studies on the PLP-dependent enzyme alanine racemase, we noted several mechanistic similarities to those found in ACPC deaminase, including autoinactivation by β-substituted D-amino acids (Badet et al., 1984; Roise et al., 1984; Walsh et al., 1981). Recently we have described time-dependent inactivation of alanine racemases isolated from Gram-positive organisms, but not Gram-negatives, by (1-aminoethyl)phosphonate isomers (Ala-P) (Badet & Walsh, 1985). Inactivation was due to the extraordinary slow dissociation behavior that resulted from tight noncovalent binding of Ala-P at the active site (Badet et al., 1986).

To test the possible efficacy of aminophosphonates as inhibitors of amino acid utilizing PLP enzymes, we have prepared the phosphonate analogue of ACPC (**1**), 1-aminocyclopropanephosphonate (ACPP) (**2**), and report herein its



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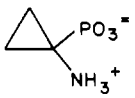

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time-dependent inhibition of ACPC deaminase from *Pseudomonas* sp. ACPC. Furthermore, time-dependent inhibition of alanine racemase from *Bacillus stearothermophilus* by ACPP is discussed.

Experimental Procedures

Materials

Rabbit muscle L-lactate dehydrogenase (LDH) (EC 1.1.1.27) (820 units/mg), porcine kidney D-amino acid oxidase (EC 1.4.3.3) (15 units/mg in 3.2 M ammonium sulfate), and HEPES were purchased from Sigma. D- and L-(1-aminoethyl)phosphonic acids were kindly provided by Dr. C. Hassal (Roche Products Ltd., Welwyn Garden City, U.K.). Dr. J. Dingwall (Ciba-Geigy) provided (1-amino-1-methylethyl)-phosphonic acid (α-methyl-Ala-P). *tert*-Butyl bromoacetate, triethyl phosphite, benzyltriethylammonium chloride, triethylamine, and diphenylphosphoryl azide were purchased from Aldrich.

Methods

Synthesis of 1-Aminocyclopropanephosphonate (**2**) *and tert-Butyl Diethylphosphonoacetate* (**3**). A solution of *tert*-butyl bromoacetate (32.9 g, 0.17 mol) and freshly distilled triethyl phosphite (28.6 g, 0.17 mol, distilled from Na) was placed in a warm oil bath (80 °C). The bath temperature was slowly raised until ethyl bromide began to distill (110 °C). After 30 min the reaction was cooled and the product distilled under vacuum (115 °C, ca. 2 mm) to afford 39.6 g (93%) of **3** as a colorless oil: ^{1}H NMR ($CDCl_3$, 60 MHz) δ 4.15 (m, 4 H), 2.80 (d, J = 20 Hz, 2 H), 1.45 (s, 9 H), 1.30 (t, J = 7 Hz, 6 H).

tert-Butyl Diethyl-1-phosphonocyclopropanecarboxylate (**4**). A suspension of benzyltriethylammonium chloride (3.60 g, 15.8 mmol) and 50% aqueous sodium hydroxide (40 mL) was treated with a solution of ester **3** (4.0 g, 15.8 mmol) and dibromoethane (20 g, 106 m mol) over 20 min. An ice–water bath was required to maintain the reaction temperature below 30 °C. After it was stirred for 1 h, the mixture was poured over CH_2Cl_2 and the aqueous layer diluted with water until all of the solid had dissolved. The aqueous layer was then extracted several more times with CH_2Cl_2. The combined organic extracts were dried (Na_2SO_4) and passed through a silica gel plug (40 mL). Removal of the solvent gave 2.26 g (51%) of cyclopropanephosphonate **4** as a colorless oil: IR (neat) 1722 cm^{-1}; ^{1}H NMR ($CDCl_3$, 60 MHz) δ 4.08 (m, 4 H), 1.45 (s, 9 H), 1.6–1.15 (m, 10 H).

Diethyl-1-phosphonocyclopropanecarboxylic Acid (**5**). *tert*-Butyl ester **4** (4.00 g, 14.4 mmol) was dissolved in cold (0 °C) freshly distilled trifluoroacetic acid (50 mL). The resulting colorless solution was stirred for 15 min at 0 °C and then concentrated under reduced pressure. Residual TFA was removed by diluting with toluene and reconcentrating (3×). The resulting colorless oil solidified upon standing to afford 3.06 g (96%) of acid **5**: mp 65–67 °C; IR ($CDCl_3$) 1710 cm^{-1}; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 4.18 (m, 4 H), 1.52 (br s, 2 H), 1.48 (m, 2 H), 1.33 (t, J = 7 Hz, 6 H).

Diethyl 1-[N-(tert-Butoxycarbonyl)amino]cyclopropanephosphonate (**6**). A solution of acid **5** (1.21 g, 5.47 mmol) and dry *tert*-butyl alcohol (25 mL) was treated with triethylamine (0.84 mL, 6.0 mmol) and diphenylphosphoryl azide (1.27 mL, 5.88 mmol). After it was heated at reflux for 5.5 h, the colorless solution was concentrated. The resulting colorless oil was dissolved in dichloromethane and extracted with water and a saturated solution of sodium bicarbonate. The combined aqueous washes were reextracted with dichloromethane. The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated to a colorless oil. Chromatography on silica gel (75% ethyl acetate/hexanes) gave 1.49 g (93%) of **6** as a colorless solid: mp 75–77 °C; IR ($CDCl_3$) 1715 cm^{-1}; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 4.96 (br s, 1 H), 4.17 (m, 4 H), 1.44 (s, 9 H), 1.5–1.33 (m, 2 H), 1.33 (t, J = 7 Hz, 6 H), 1.10 (m, 2 H).

1-Aminocyclopropanephosphonate (**2**). Diethyl phosphonate **6** (750 mg) in 6 N HCl (50 mL) was heated to 100 °C for 24 h and then concentrated under vacuum. The resulting colorless oil was dissolved in absolute ethanol (12 mL) and treated with propylene oxide (0.5 mL). After standing overnight at room temperature, the mixture was filtered to obtain 348 mg (100%) of ACPP (**2**) as colorless crystals: mp 260–261 °C: IR (Nujol) 1620, 1550, 1155, 1045, 1015, 940, 880, 780, 625 cm^{-1}; ^{1}H NMR (D_2O, pH 2, 250 MHz) δ 1.05 (m, 2 H), 0.95 (m, 2 H); ^{13}C NMR (D_2O, pH 5.5) δ 31.3 (d, J = 186 Hz), 10.3.

Synthesis of ACPC (**1**). ACPC was synthesized by the seven-step route reported by Hiyama and Kai (1982; Kakimoto et al., 1982). 1,8-Diazabicyclo[5.4.0]undec-7-ene was used to convert methyl 2,3-diazidocarboxylate to methyl 2-azidoacrylate.

Enzymes. 1-Aminocyclopropanecarboxylate deaminase from *Pseudomonas* sp. ACPC was purified to homogeneity as described by Honma and Shimomura (1978). From 9 g of wet cells a total of 14 mg of deaminase was obtained with a specific activity of 9.4 μmol·min^{-1}·mg^{-1} and an A_{278}/A_{418} ratio of 5–6. Pure *Bacillus stearothermophilus* alanine racemase was obtained from Dr. B. Badet of these laboratories (Inagaki et al., 1986).

Protein Determination. Protein concentrations were determined by the method of Bradford (1976). Lysozyme and bovine serum albumin were used as standards.

Spectrophotometric Determination. UV–visible spectra were recorded on either Perkin-Elmer Model 554 or lambda 5 instruments. Proton NMR spectra were recorded on either

a 60-MHz Varian T-60 or a 250-MHz Bruker WM250 instrument. ^{13}C NMR spectra were recorded on a 270-MHz Bruker WM270 instrument operating at 25.1 MHz. Infrared spectra were obtained on a Perkin-Elmer 283B spectrometer. CD spectra were recorded on a AVIV spectropolarimeter, Model 60 DS.

Assays. Enzymatic activity of the deaminase was monitored spectrophotometrically by using the previously described lactate dehydrogenase (LDH) coupled assay (Walsh et al., 1981). One unit of activity is defined as that amount of enzyme required to convert 1 μmol of NADH to NAD^+ per minute at 18 °C. The standard 1.0-mL assay was at 18 °C and contained 50 mM potassium pyrophosphate (pH 7.8), 155 units of L-lactate dehydrogenase, 0.3 mM NADH, 20 mM ACPC, and 10–20 μg of ACPC deaminase. The reaction was initiated by the addition of enzyme and was monitored by the decrease in the NADH 340-nm absorbance.

Enzymatic activity of the racemase was monitored spectrophotometrically using the coupled assay described by Badet et al. (1984). One unit of activity is defined as that amount of enzyme required to epimerize 1 μmol of alanine per minute at 37 °C. The assay mixture was at 37 °C and contained 100 mM HEPES (pH 7.4), 16 units of D-AAO, 155 units of LDH, 0.3 mM NADH, 30 mM L-Ala, and alanine racemase. The reaction was initiated by the addition of enzyme and was monitored by the decrease in the NADH 340-nm absorbance.

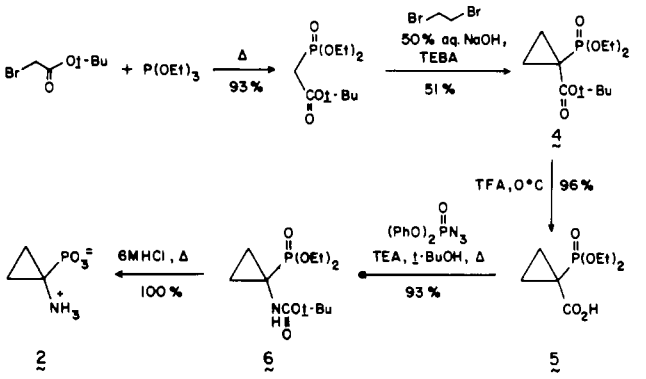
^1H NMR Assay for ACPC Deaminase Turnover of ACPP. An attempt at detecting turnover of ACPP by ^1H NMR was performed by monitoring a 37 °C 50 mM potassium pyrophosphate (0.4 mL; pD 8.8) solution containing ACPP (2.0 mg, 11.5 μmol), ACPC deaminase (102 μg), and an internal integration standard, sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]-propionate (TSP) (0.25 mg) for 48 h. After 2, 5, 24, and 48 h a 250-MHz ^1H NMR spectrum was recorded. The conversion of ACPC (4.7 mg, 46.5 μmol) to α-ketobutyrate was monitored under identical conditions.

Inactivation Assays for ACPC Deaminase. The rate of approach to steady-state inhibition k_{obsd} was determined by adding 13 μg of deaminase to the LDH-coupled assay mixture at several concentrations of ACPC and ACPP. Velocities at various times were determined from the slope of the resulting progress curves. A plot of $\ln [(v - v_s)/(v_0 - v_s)]$ vs. time, where v_s, v_0, and v are the velocities at steady-state, time zero, and time t, respectively, gave a line with the slope equal to the apparent first-order rate constant ($-k_{obsd}$).

The rate of recovery of catalytic activity (k_2 or k_4) was determined by preincubating deaminase (128 μg) in 50 mM potassium phosphate buffer (pH 7.6) containing 85 μM ACPP. After 60 min at 10-μL aliquot was removed and added (100-fold dilution) to the LDH-coupled assay containing 15 mM ACPC. The resulting increase in the rate of NADH consumption was monitored until a steady-state velocity was reached, which was then used in the plot of $\ln [100 - (v/v_s)]$ vs. t. The control rate was obtained by addition of 13 μg of deaminase directly to the same assay mixture.

Inactivation Assays for Alanine Racemase. The experimental conditions of Badet et al. (1986) were followed for the study of ACPP inactivation of alanine racemase isolated from *Bacillus stearothermophilus*. The reversible K_i for alanine racemase was calculated from the progress curves generated by addition of racemase to a 37 °C assay solution containing ACPP. Preincubation of racemase with ACPP at 37 °C (pH 7.4) for 24 h followed by a 100-fold dilution into an assay solution containing 30 mM L-alanine gave the residual activity used in the calculation of K_i*.

Scheme II: Synthesis of ACPC



Kinetic Calculations. All K_m, V_{max}, and K_i values were calculated by using v^4 weighting (Wilkinson, 1961).

ACPP-Induced Change in the Optical Spectrum of Deaminase. A solution of deaminase (512 μg) in 100 mM potassium phosphate (pH 7.5) was cooled to 18 °C, and the optical spectrum was recorded between 280 and 500 nm. ACPP was then added to a final concentration of 15–90 μM, and the spectrum was recorded. The rate of the λ_{max} shift (418 to 333 nm) was monitored continuously at 418 nm. The rate of approach to steady state was determined by plotting $\ln [(A - A_s)/(A_0 - A_s)]$ vs. time, where A, A_s, and A_0 are the 418-nm absorbance at times t, steady state, and zero. The slope of the resulting line is equal to the apparent first-order rate constant ($-k_{obsd}$).

Circular Dichroism Studies. A circular dichroism spectrum was recorded for native enzyme in 100 mM KP_i (pH 7.5) at 4 °C and for enzyme preincubated with 250 μM ACPP at 4 °C for 30 min. CD spectra were recorded at 4 °C in 1-mm cells.

Attempted $NaBH_4$ Reduction of the ACPP–Deaminase Complex. A preincubated (for 10 min) colorless 18 °C solution containing 100 mM potassium phosphate solution (0.5 mL, pH 7.5), 250 μM ACPP, and 640 μg of ACPC deaminase was treated with 100 μg of $NaBH_4$ in 10 μL of 10% aqueous sodium carbonate, and after 20 min a spectrum was recorded. Excess $NaBH_4$ was quenched by addition of 2 μL of a 1% PLP solution. The percent of the enzyme with reduced PLP was determined by transferring a 10-μL aliquot of this mixture or of the mixture prior to treatment with PLP to a 37 °C LDH-coupled assay mixture and measuring the steady-state velocity. The control rate was determined from ACPP-treated ACPC deaminase under identical conditions except for the addition of $NaBH_4$. The amount of pyridoxamine phosphate could be estimated from the UV–vis spectrum obtained after exhaustive dialysis of the mixture against 100 mM potassium phosphate. ACPC deaminase treated with $NaBH_4$ in the absence of ACPP followed the same protocol.

Results and Discussion

Preparation and Properties of ACPP (**2**). As shown in Scheme I, the synthesis of ACPP began with the preparation of phosphonate **3** from an Arbuzov reaction between triethyl phosphite and *tert*-butyl bromoacetate. Alkylation of **3** with dibromoethane under phase-transfer conditions gave the cyclopropylphosphonate **4** in 51% yield (Singh & Danishefsky, 1975). After deprotection, acid **5** was converted to ACPP by a Curtius rearrangement using diphenylphosphoryl azide (Shiorri et al., 1972) followed by hydrolysis in 6 N HCl. During the course of our studies on ACPP inactivation of ACPC deaminase, Diel and Maier published a synthesis of ACPP by a different route (Diel & Maier, 1984). (See Scheme II.)

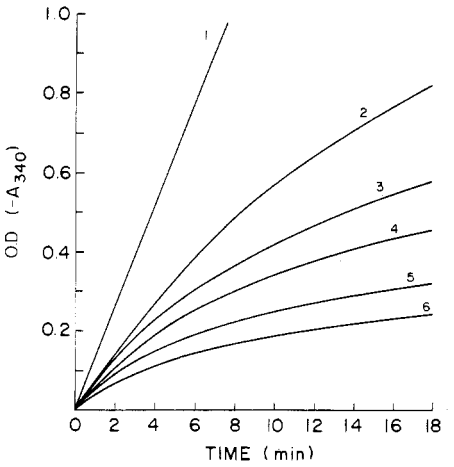


FIGURE 1: Deaminase assay progress curves generated from addition of deaminase (13 μg) to a LDH-coupled assay mixture containing ACPC (5 mM) and 0, 50, 75, 100, 150, and 200 μM ACPP concentrations (curves 1, 2, 3, 4, 5, and 6, respectively).

Test for Ring Opening of ACPP. The facile conversion of ACPC to α-ketobutyrate by ACPC deaminase suggested that the structurally and electronically similar phosphonate analogue, ACPP (**2**), might be converted to the corresponding α-ketobutylphosphonate. However, in contrast to ACPC, incubation of ACPC deaminase with ACPP under the LDH-coupled assay conditions showed no loss in the 340-nm NADH absorbance. Since the expected product, 2-ketobutylphosphonate, may not be a substrate of LDH, we analyzed for the ability of deaminase to process ACPP by proton NMR. No detectable loss (<1%) of ACPP resonances occurred in 24 h under conditions where ACPC was completely converted to α-ketobutyrate in less than 2 h. Thus, if ACPC deaminase does catalyze the ring cleavage of ACPP, the rate must be exceedingly slow.

Inhibition of ACPC Deaminase by ACPP. The inhibitory effect of ACPP on the rate of ACPC turnover was revealed when deaminase was added to LDH-coupled assay mixtures containing ACPC ($2K_m$) and ACPP (Figure 1). The resulting nonlinear progress curves clearly showed that the presence of ACPP produced a time-dependent inhibition of deaminase activity. In addition, the progress curves showed that a steady-state velocity is attained slowly on the time scale of the assay and that the final velocity depended on inhibitor concentration. These results suggested that the time-dependent loss in activity was not due to irreversible enzyme inactivation, substrate depletion, or product inhibition but rather to slow-binding inhibition, a class of inhibition that is currently receiving considerable attention (Morrison & Walsh, 1987).

Equally apparent from Figure 1 was that very low concentrations of ACPP inhibit ACPC deaminase catalysis. An accurate calculation of a final inhibitory constant (K_i) under these assay conditions (18 °C), however, was complicated by the fact that at low concentrations of ACPP the onset of inhibition was so slow that the NADH is depleted prior to establishing steady-state equilibrium. Preincubation of ACPP and deaminase followed by addition of ACPC also did not enable an accurate K_i determination due to some loss in enzymatic activity that apparently occurs during the period required to establish the steady-state velocity. In contrast, a reproducible K_i determination was possible if the assay temperature was raised from 18 to 37 °C. At 37 °C the time required to reach steady state was shortened to 2–3 min during which a decrease in velocity due to substrate depletion or thermal instability was insignificant. The Lineweaver–Burk plot showed that ACPP was a competitive inhibitor of ACPC deaminase with a K_i of 6.9 μM (data not shown). The 3 mM K_m determined for ACPC gives a K_m/K_i ratio of ca. 500, a value that points clearly to the potency of the phosphonate substrate analogue as an inhibitor of ACPC deaminase.

Scheme III: Possible Slow-Binding Mechanisms[a]

$$E + I \underset{k_2}{\overset{k_2}{\rightleftharpoons}} E\cdot I \quad \text{(A)}$$

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot I^* \quad \text{(B)}$$

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot I^* \quad \text{(C)}$$

[a] $K_i = k_2/k_1$; $K_i^* = K_i k_4/(k_3 + k_4)$.

Analysis of Time-Dependent Inhibition Behavior. A slow-binding inhibitor has been defined as an inhibitor that induces a slow onset of activity loss relative to the rate of diffusion (Morrison & Stone, 1985). Several examples of slow-binding inhibitors have been described in the literature (Morrison & Walsh, 1987). Three mechanisms have been proposed to describe slow onset of inhibition (Cha, 1975, 1976; Morrison, 1982) (see Scheme III). These kinetically distinct mechanisms are labeled A, B, and C and respectively involve a slow initial interaction between the inhibitor and the enzyme (A), a rapid association to form an E·I complex followed by a slow unimolecular change to E·I* (B), and a slow initial isomerization of the enzyme followed by rapid binding of the inhibitor (C).

These mechanisms may be kinetically differentiable if the following conditions are satisfied (Cha, 1975): (1) the concentration of enzyme is much lower than the substrate, (2) only a small percentage of the substrate is converted to product during the course of the assay, (3) the inhibitor concentration does not significantly change upon formation of the enzyme–inhibitor complex, and (4) the decrease in velocity is represented by

$$v = v_s + [(v_0 + v_s)\exp(-k_{obsd}t)] \quad (1)$$

where v_s, v_0, and v are the steady-state, initial, and time t velocities, respectively, and k_{obsd} is the apparent first-order rate constant for the establishment of the steady-state equilibrium. From eq 1, a plot of $\ln [(v - v_s)/(v_0 - v_s)]$ vs. time will be linear and have a slope equal to $-k_{obsd}$.

Thus, the three slow-binding mechanisms can be differentiated by the relationships between k_{obsd}, [I], and [S]. The k_{obsd} predicted by mechanisms A, B, and C is given by eq 2, 3, and 4, respectively, where $K_i' = K_i(1 + [S]/K_m)$.

$$\text{(A)} \quad k_{obsd} = k_2 + k_1[I]/(1 + [S]/K_m) \quad (2)$$

$$\text{(B)} \quad k_{obsd} = k_4 + k_3[I]/([I] + K_i') \quad (3)$$

$$\text{(C)} \quad k_{obsd} = k_1/(1 + [S]/K_m) + k_2K_i/([I] + K_i) \quad (4)$$

To determine which mechanism best accounts for the slow inhibitory behavior observed in this case, we added deaminase to LDH-coupled assay mixtures at several concentrations of ACPC and ACPP and then measured the reaction velocities at various times from the resulting progress curves. Figure 2 shows that the plot of $\ln [(v - v_s)/(v_0 - v_s)]$ vs. time at several ACPP concentrations is linear through several half-lives and that the slope ($-k_{obsd}$) increases with increasing inhibitor concentration. This correlation is consistent with mechanisms A and B (eq 2 and 3) but not mechanism C, since eq 4 predicts an increase in k_{obsd} with decreasing [I].

In principle, mechanisms A and B can be differentiated by the following three criteria (Shapiro & Riordan, 1984): (1) the effect of [I] on v_0; (2) a plot of $1/(k_{obsd} - k_{rgn})$ vs. $1/[I]$, where k_{rgn} is the rate of regain of activity upon dilution of fully inhibited enzyme; and (3) a plot of $1/(k_{obsd} - k_{rgn})$ vs. [S] at

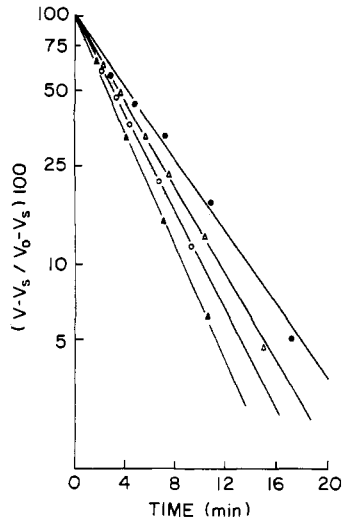


FIGURE 2: Determination of k_{obsd} for time-dependent inhibition of ACPC deaminase by ACPP. Velocities measured from progress curves generated by addition of deaminase (13 μg) to a LDH-coupled assay mixture containing 5 mM ACPC and ACPP at 50 (●), 75 (△), 100 (○), and 150 μm (▲) concentrations. k_{obsd} was determined from the slope of the lines.

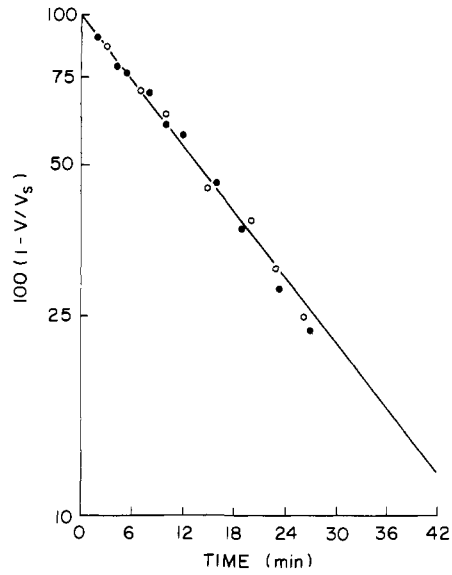


FIGURE 3: Reversal of ACPP inhibition of ACPC deaminase by dilution of ACPC deaminase (128 μg) that was preincubated in 85 μM ACPP (100 μL, 50 mM KP_i, pH 7.6) for 1 h at 18 °C. A 10-μL aliquot was then removed and added to a 18 °C solution of assay mixture containing ACPC (15 mM). k_{rgn} is calculated from the slope of the line.

constant [I]. Thus, in an attempt to differentiate between mechanisms A and B, these three criteria were each examined, and the results are discussed in turn.

(*1*) *Effect of* [*I*] *on* v_0. Mechanism A predicts that the initial velocity will be independent of inhibitor concentration, whereas mechanism B predicts that the initial velocity will decrease with increasing inhibitor concentrations due to the rapid initial formation of the intermediate E·I complex. The progress curves shown in Figure 1 have initial velocities that decrease with increasing inhibitor concentrations. This result supports mechanism B, but the nonlinearity of the progress curves from the outset implies that at least some of the decrease in the initial velocity was due to the presence of the slow-forming enzyme–inhibitor complex. This failure to completely separate the two inhibition phases prevented an accurate measurement of v_0 and thereby a fully conclusive argument against mechanism A based on this criterion.

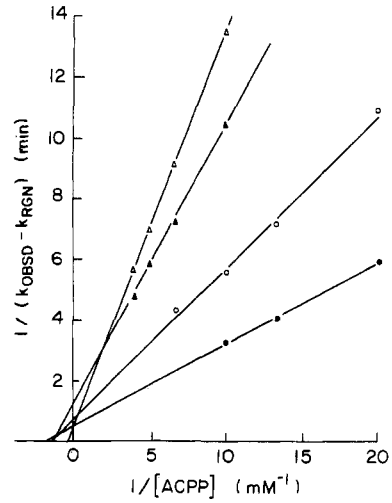


FIGURE 4: Plots of $1/(k_{obsd} - k_{rgn})$ vs. $1/[ACPP]$ at ACPC concentrations of 2.5 (●), 5 (○), 10 (▲), and 15 mM (△).

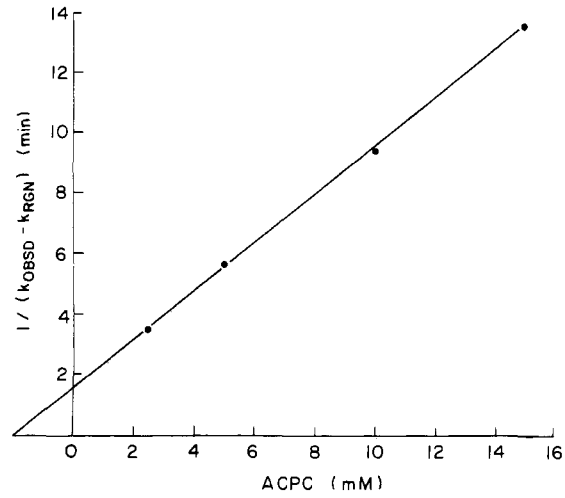


FIGURE 5: Plot of $1/(k_{obsd} - k_{rgn})$ vs. [ACPC] at 100 μM ACPP.

(*2*) *Plot of* $1/(k_{obsd} - k_{rgn})$ *vs.* $1/[I]$. This criterion requires measurement of k_{rgn}, which is the rate of regain in enzymatic activity upon dilution of fully inhibited enzyme into the assay mixture containing 15 mM ACPC ($6K_m$) and is equal to k_2 in mechanism A and k_4 in B. The resulting progress curve had an initial velocity of near 0 but returned to a final velocity of 82% of the control. The plot shown in Figure 3 of ln [100 − (v/v_s)] vs. t, where v and v_s are the velocities at time t and at inhibition equilibrium, was linear through two half-lives and had a slope ($-k_{rgn}$) of 0.051 min^{-1} ($t_{1/2}$ = 13.5 min). By use of this k_{rgn}, the plot of $1/(k_{obsd} - k_{rgn})$ vs. $1/[I]$, shown in Figure 4, gave an x intercept near the origin. This would permit mechanism A, but it would also be consistent with the predicted x intercept ($-1/K_i$) for mechanism B if the initial inhibition constant is large in comparison with inhibitor concentrations used in the assay. Indeed, these data would suggest by mechanism B an initial K_i of ca. 1 mM for ACPP, a value in the range of the 3 mM K_m for the substrate ACPC.

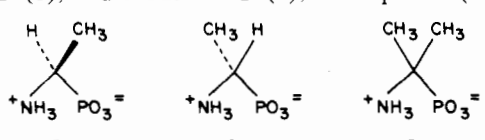
(*3*) *Plot of* $1/(k_{obsd} - k_{rgn})$ *vs.* [*S*] *at Constant* [*I*]. The final kinetic criterion for differentiating mechanisms A and B is a plot of $1/(k_{obsd} - k_{rgn})$ vs. [S] at constant [I]. Mechanism A predicts a line with an x intercept at the K_m of ACPC while mechanism B predicts an x intercept of $[-K_m([I] + K_i)]/K_i$. The plot shown in Figure 5 has an x intercept of 2 mM, a value close to the 2.5 mM K_m determined for ACPC. Again, these data can support either mechanism A or mechanism B with a large initial K_i ($K_i \gg [I]$) (e.g., if K_i for ACPP is ca. 1 mM,

then the x intercept would be 2.75 mM).

Suppose the monophasic kinetics observed for ACPC inhibition of deaminase is most simply represented by mechanism A. Observation of slow inhibition therefore would imply that the rate (k_{obsd}) of combining E and I to form E·I is slow on the steady-state time scale. Since k_{obsd} is equal to $k_1[I]$, the slow onset of inhibition could arise either from a low concentration of a classical competitive inhibitor ($k_1 \sim 10^8$ M^{-1} s^{-1}) or from a slow bimolecular rate constant (k_1) relative to diffusion (10^8 M^{-1} s^{-1}) for the binding of an inhibitor to the enzyme. The bimolecular rate constant (k_1) of 100 M^{-1} s^{-1} calculated for mechanism A from the slopes of the lines shown in Figure 4 would indicate that a remarkably slow association rate accounts for the slow onset of inhibition observed here. This value for k_1 gives a K_i (k_2/k_1) of 5.8 μM, which is consistent with the 6.9 μM K_i determined above. The molecular basis of an association rate so much slower than diffusion control would be unclear but may imply some set of energy barriers that must be surmounted to align the inhibitor and active site during binding.

To date, nearly all reported examples of slow-binding inhibition (Morrison & Walsh, 1986) exhibit biphasic inhibition kinetics due to the formation of an initial E·I complex followed by equilibrium conversion to the E·I* complex. One exception reported earlier is the monophasic slow-binding inhibition of adenosine deaminase and adenylate deaminase by coformycin (Frieden & Kurz, 1980). Despite a limiting association rate constant of 10^4–10^5 M^{-1} s^{-1} (2–3 orders of magnitude higher than this ACPC deaminase case), an intermediate E·I was not detected even with stopped-flow kinetic techniques. These results are similar to the results presented here, and although the data are consistent with mechanism A, they cannot rule out mechanism B, where the initial binding of the inhibitor to the active site is very weak. In this case, mechanism B is characterized by monophasic kinetics because the initial weakly associated E·I complex is present in the inhibition assays at kinetically insignificant concentrations and thereby escapes detection ($[I] \ll K_i$). Higher concentrations of ACPP would favor E·I formation, but not higher E·I concentrations, since there would be a corresponding increase in the rate ($k_3[I]$) of conversion of E·I to the more stable E·I*. A k_3 of between 0.014 and 0.03 s^{-1} is calculated from the y intercepts of the plots shown in Figure 4. Thus it would appear that the slow-binding inhibition kinetics observed here arise from either a dramatically slow initial encounter of inhibitor and the active site (mechanism A) or a slow realignment of a very weakly associated E·I (K_i = mM) complex (a special case of mechanism B) to a tightened E·I* complex (K_i^* = μM). Given the detection of a slow conversion of initial E·PLP 418-nm chromophore to a 333-nm chromophore (see later section) which parallels enzyme inactivation, mechanism B seems more likely.

Inhibition of Alanine Racemase by ACPP and Inhibition of ACPC Deaminase by Ala-P. Our interest in studying the generality of α-aminophosphonate inhibition of PLP-dependent enzymes led us to look at the inhibition of alanine racemase with ACPP and of ACPC deaminase with (α-aminoethyl)-phosphonates (Ala-P). Previous work (Badet & Walsh, 1985) found the three phosphonate analogues of alanine, L-Ala-P (**7**), D-Ala-P (**8**), and α-Me-Ala-P (**9**), to be potent (K_m/K_i =



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4000) slow-binding inhibitors of the PLP-dependent alanine racemase isolated from Gram-positive organisms. In comparison to the kinetics observed for ACPP inhibition of ACPC deaminase, Ala-P inactivation of racemase follows mechanism B as supported by the complete separation of the initial (E·I) and final (E·I*) inhibition phases. Another difference was the 500-fold slower rate of regain in activity, which led to nearly irreversible binding of Ala-P to the racemase ($t_{1/2}$ = 115 h).

Table I: Potency of ACPP as a Slow-Binding Inhibitor of Two PLP Enzymes

enzyme	K_m/K_i	K_i^* (μM)	$t_{1/2}$(regain)
ACPC deaminase	500	5–7	14 min
alanine racemase	2000	2	>1 day

The structural and electronic similarity of ACPP and Ala-P suggested that ACPP may, like Ala-P, be a potent inhibitor of the antibiotic target alanine racemase. The results of our study showed that ACPP was indeed a potent (K_m/K_i = 2000) slow-binding inhibitor of alanine racemase. The kinetics were similar to Ala-P inactivation of racemase with an easily detectable initial enzyme–inhibitor complex (K_i = 8 mM) and a slow-forming tightly associated (K_i^* = 2 μM) E·I* complex (data not shown). The large K_i^*/K_i ratio implies a very small k_4, and thus ACPP, like Ala-P, is an effective active-site-directed irreversible inhibitor of alanine racemase, by virtue of slow dissociation. (See Table I.)

In contrast to alanine racemase, ACPC deaminase can apparently discriminate between ACPP and Ala-P, since L-Ala-P (**7**), D-Ala-P (**8**), and α-Me-Ala-P (**9**) were all just weak classical competitive inhibitors with no slow-binding properties (K_i's of 0.45, 4.5, and 21.5 mM, respectively). The dramatic difference in inhibition behavior between ACPP and Ala-P is surprising in light of the similarities in electronic and steric features. The larger N–C–P bond angles expected in ACPP (Wiberg & Ellison, 1974; Wertz & Allinger, 1974) may account for the tighter binding of ACPP to deaminase, possibly as a result of an interaction that stabilizes a higher energy protein conformation.

Effects of ACPP and Ala-P on ACPC Deaminase UV–Vis Spectrum. The kinetic data analyzed in the previous sections showed that ACPP is a slow-binding inhibitor of both ACPC deaminase and alanine racemase activity but failed to shed any light on the molecular basis for the slow onset of inhibition. The bound PLP coenzyme present in these enzymes offers a prominent optical probe of the active-site microenvironment during the course of the E to E·I transition and thereby should aid in a better understanding of this behavior and in future development of slow-binding inhibitors of pharmacologically important enzymes. In the initial effort, Badet et al. (1986) found that the UV–vis spectrum for alanine racemase remained unaltered during incubation with Ala-P. ACPC deaminase, however, when treated with ACPP did result in a time-dependent shift in the PLP chromophore λ_{max}. As illustrated in Figure 6A, the absorption shift was from 418 nm for native enzyme to 333 nm for fully inhibited enzyme with the extent of the conversion dependent on [I] (Figure 6B).

The rate of conversion of the 418- to 333-nm absorbance was determined by continuously monitoring the decrease in the 418-nm absorbance. A plot of $\ln [(A - A_s)/(A_0 - A_s)]$ vs. time, where A, A_s, and A_0 are the 418-nm absorbance at time t, at the inhibition equilibrium, and at time zero, respectively (Figure 7), gave a line with a slope equal to the apparent first-order rate constant $-k_{obsd}$. The $1/(k_{obsd} - k_{rgn})$ value at 100 μM ACPP was close to the y intercept in the plot of $1/(k_{obsd} - k_{rgn})$ vs. [S] shown in Figure 5. A plot of $1/(k_{obsd} - k_{rgn})$ vs. $1/[I]$ (Figure 7) gave an inactivation rate (k_i) of

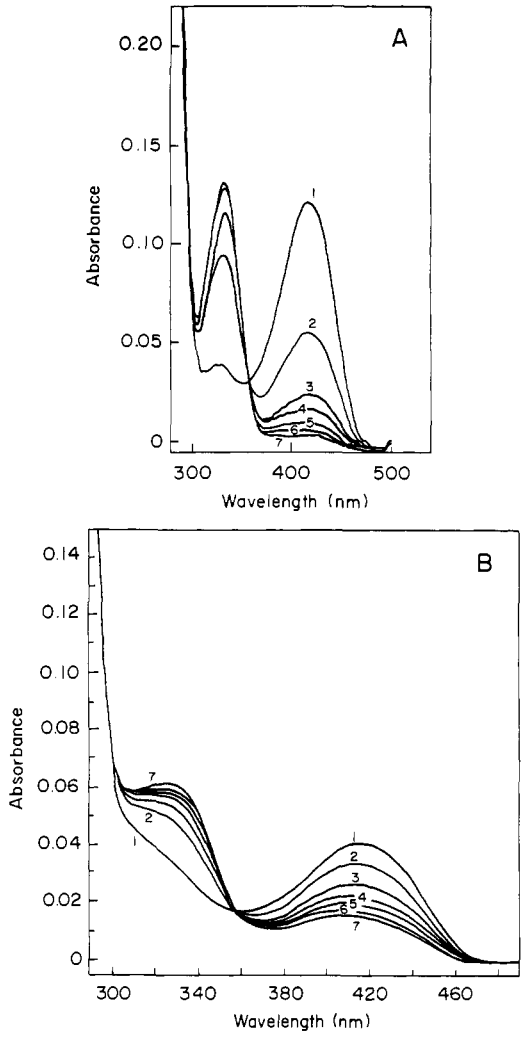
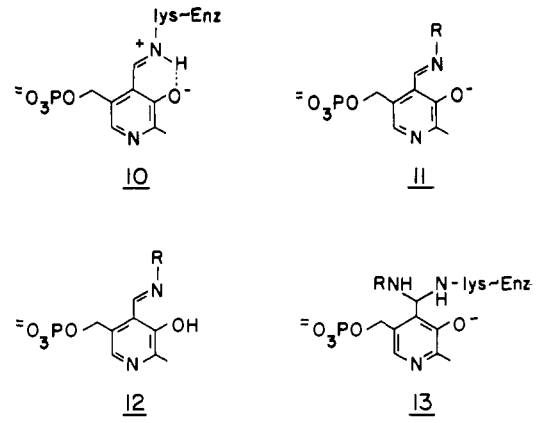


FIGURE 6: UV–vis spectra of ACPC deaminase (1.3 mg/mL) at 18 °C in 100 mM potassium phosphate (pH 7.5). (A) Spectra were recorded at steady state after preincubation (10–30 min) with 0, 15, 30, 45, 60, 90, and 200 μM ACPP (curves 1, 2, 3, 4, 5, 6, and 7, respectively). (B) Time-dependent inhibition spectra were recorded before (curve 1) and after (curves 2–7) treatment of enzyme (0.57 mg/mL) with ACPP (25 μM final concentration) at a scan rate of 120 nm/min and at the following times: 0, 0.7, 3, 8, 12, 20, and 35 min (curves 1, 2, 3, 4, 5, 6, and 7, respectively).

150 M^{-1} s^{-1}, a value very close to the k_1 of 100 M^{-1} s^{-1} obtained from the inhibition assays. Thus, the rate at which the 418-nm absorbance is dissipated corresponds directly to the slow approach to the equilibrium velocity observed in the inhibition assays.

The shift in the λ_{max} for the PLP chromophore signifies a change in the electronic structure of the PLP coenzyme and should therefore be particularly useful in an investigation of the slow-binding behavior observed in this case. On the basis of reported spectra for the various ionic forms of PLP–amine adducts (Metzler et al., 1980; Kallen et al., 1985), the 418-nm absorbance found in the native enzyme is consistent with the imine (**10**) formed between the PLP and the ϵ-amino group of an active-site lysine residue, whereas the λ_{max} of 333 nm found at inhibition equilibrium corresponds to at least the following four possibilities: (1) pyridoxamine phosphate (PMP); (2) the deprotonated imine **11**, which is apparently present in aspartate aminotransferase at high pH (Davis & Metzler, 1972); (3) the enolimine **12**, which is the favored tautomer in hydrophobic environments and is though to be present in phosphorylase (Shaltiel & Cortijo, 1970; Johnson et al., 1970); and (4) the *gem*-diamine **13**, which may account



for the 333-nm absorbance seen with glutamate decarboxylase at high pH (O'Leary, 1971). Since PMP is unlikely to be present based on the kinetic and turnover data and since imine **11** is present only at high pH, the conversion of the 418-nm absorbance to 333 nm most likely arises either from a slow protein conformational change that moves the PLP to a more hydrophobic environment or from slow formation of a *gem*-diamine, possibly with the participation of the active-site lysine.

Next we looked to see if the structurally similar but kinetically different aminophosphonate, L-Ala-P, produced a similar change in the UV–vis spectrum of deaminase. The result was an immediate and identical absorbance shift at concentrations of $10K_i$. This suggests that although the rate of approach to the inhibition equilibrium may differ between ACPP and Ala-P inhibition, the same PLP–α-aminophosphonate adduct is generated.

Effects of ACPP on the CD Spectrum of ACPC Deaminase. Circular dichroism spectra of PLP–enzymes, such as alanine racemase (Badet et al., 1986) and L-aspartate transaminase (Martinez-Carrion et al., 1970), have been reported to be indicative of the PLP microenvironment (Kallen et al., 1985). To examine further the changes that occur in ACPC deaminase during inhibition by ACPP, we recorded a CD spectrum for native ACPC deaminase and deaminase after preincubation with ACPP (data not shown) and observed the same 418- to 333-nm absorbance shift in the PLP λ_{max} observed in the UV–vis spectra, albeit with a shift from positive (418 nm) to negative (333 nm) rotation. Although the significance of the inversion in the optical rotation sign is unclear, the optical activity associated with the 333-nm absorbance implies that the PLP remains in a chiral environment in the steady-state complex. Furthermore, the similarity of the aromatic amino acid absorption region in E and in E·I* (250–290 nm) may imply that a slow protein conformational change is not involved in the E to E·I* transition.

Attempted Reduction of ACPP-Inhibited ACPC Deaminase. The UV–vis data suggested that the PLP coenzyme in the inhibited enzyme complex exists either as imine **12** or as *gem*-diamine **13**. To probe the mode of binding of ACPP to the PLP coenzyme, we tested for imine **12** by first adding sodium borohydride to ACPC deaminase that had been preincubated with ACPP ($>10K_i$) and then measuring any resulting loss of enzymatic activity and production of PMP. The data clearly showed that ACPC deaminase preincubated with ACPP was resistant to borohydride reduction (20 min) whereas the native enzyme underwent instantaneous reduction of the PLP cofactor as observed by the conversion of the 418-nm λ_{max} to the expected 325-nm absorbance of PMP and by the loss of all enzymatic activity. In contrast, ACPP-treated ACPC deaminase retained 84% of the activity of deaminase treated with ACPP but not with $NaBH_4$ (see Methods).

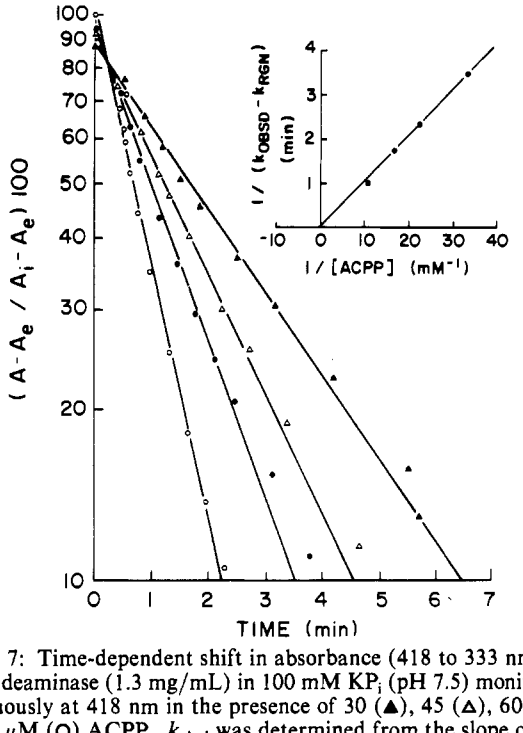


FIGURE 7: Time-dependent shift in absorbance (418 to 333 nm) of ACPC deaminase (1.3 mg/mL) in 100 mM KP_i (pH 7.5) monitored continuously at 418 nm in the presence of 30 (▲), 45 (△), 60 (●), and 90 μM (○) ACPP. k_{obsd} was determined from the slope of the lines. (Inset) Double-reciprocal plot of $1/(k_{obsd} - k_{rgn})$ vs. $1/[ACPP]$, where k_{rgn} is 0.051 min^{-1}.

Furthermore, extensive dialysis of the $NaBH_4$-treated inhibitor–enzyme complex returned the 418-nm absorbance in the UV–vis spectrum to nearly that of the native enzyme and thereby showed that only a small percentage of the PLP was converted to PMP under these conditions.

Although the failure of sodium borohydride to reduce the ACPP–deaminase complex might be construed as evidence for the geminal diamine, we note that sodium borohydride has also failed to reduce alanine racemase treated with Ala-P even though there was no change in the PLP chromophore in the fully inhibited form and thereby no *gem*-diamine intermediate (Badet et al., 1986). In that case the lack of reduction was postulated to be a result of a protein conformational change that hinders borohydride accessibility to the active site.

CONCLUSIONS

ACPP was shown to be a potent slow-binding slow-dissociating inhibitor of the cyclopropyl-cleaving PLP-dependent enzyme ACPC deaminase and the PLP-dependent enzyme alanine racemase. Biphasic kinetics were observed for racemase inhibition indicating the kinetically significant participation of an intermediate E·I complex prior to the steady-state E·I* complex. Deaminase, on the other hand, exhibited monophasic inhibition characterized by a slow bimolecular rate constant (k_1) of 100 M^{-1} s^{-1}. A net association rate constant 10^6-fold slower than that for diffusion suggested either that an energy barrier existed for initial inhibitor binding to enzyme or (more likely) that a weakly associated E·I (K_i = mM) did form prior to the steady-state E·I* (K_i = μM) complex but was undetectable under the assay conditions.

In an effort to probe further the slow-binding inhibition of deaminase, we monitored the PLP microenvironment by UV–vis and CD spectroscopies. In each case addition of ACPP resulted in a shift of the PLP chromophore from 418 to 333 nm. The rate of the absorbance shift was consistent with the rate constants calculated in the inhibition assays. The shift is likely a result of a change in the favored PLP tautomeric form or formation of a stable hemiaminal by addition of the amino group of ACPP to the aldimine carbon of the resting PLP imine. Although no definitive proof for either explanation is forthcoming, the failure of sodium borohydride to reduce the fully inhibited enzyme is most easily explained by formation of a hemiaminal.

Finally, the dramatic potency of ACPP toward ACPC deaminase and toward Gram-positive alanine racemases suggests further investigation on ACPP and its effect on other PLP–enzymes. An interesting possibility is in ethylene biosynthesis, where ACPP might inhibit ethylene production by preventing the PLP-linked ACPC synthase from converting *S*-adenosylmethionine to ACPC. The O_2-requiring enzyme(s) that convert ACPC to ethylene and cyanide are not demonstrably inhibited by ACPP (S. F. Yang, private communication).

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Phosphonate Analogues of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate as Substrates or Inhibitors of Procaryotic and Eucaryotic Enzymes Degrading Dinucleoside Tetraphosphates†

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ABSTRACT: The substrate specificity of procaryotic and eucaryotic AppppA-degrading enzymes was investigated with phosphonate analogues of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA). App(CH_2)ppA (I), App(CHBr)ppA (II), and Appp(CH_2)pA (III), but not Ap(CH_2)pp(CH_2)pA (IV), are substrates for lupin AppppA hydrolase (EC 3.6.1.17) and phosphodiesterase I (EC 3.1.4.1). None of the four analogues is hydrolyzed by bacterial AppppA hydrolase (EC 3.6.1.41), and only analogue III is degraded by yeast AppppA phosphorylase (EC 2.7.7.53). The analogues are competitive inhibitors of all four enzymes. The affinity of analogue IV is 3–40-fold lower than that of analogues I–III for all four enzymes. Introduction of one methylene (as in I and III) [or bromomethylene (as in II)] group into AppppA results in a 3–15-fold increase of its affinity for lupin and *Escherichia coli* AppppA hydrolases. The same modifications only negligibly (10–30%) affect its affinity for yeast AppppA phosphorylase and decrease its affinity for lupin phosphodiesterase I about 2.5-fold. The data provide further evidence for the heterogeneity among catalytic sites of all four AppppA-degrading enzymes.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA), discovered as a product of the back-reaction of aminoacyl-tRNA synthetase (Zamecnik et al., 1968), has subsequently turned out to be a ubiquitous dinucleotide present in all cell types investigated (Zamecnik, 1983; Garrison & Barnes, 1984). The dinucleotide is synthesized in vitro by some aminoacyl-tRNA synthetases from several organisms (Zamecnik et al., 1968; Plateau et al., 1981; Goerlich et al., 1982; Jakubowski, 1983). Presumably the same enzymes are involved in AppppA synthesis in vivo, although this remains to be confirmed. A variety of factors affect the cellular level of AppppA, and on the basis of this, two hypotheses conce ning the function of AppppA in cells have been advanced. One of them proposes that AppppA is a positive pleiotropic activator that modulates DNA

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