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AMINOOXYPHOSPHONATES AS SLOW BINDING INHIBITORS OF ASPARTATE AND ALANINE AMINOTRANSFERASES FROM PORCINE HEART

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Aminooxymethylphosphonic (AOMP), 1-aminooxyethylphosphonic (1-AOEP) and 2-aminooxyethyl-phosphonic (2-AOEP) acids have been synthesised and were found to be potent slow binding inhibitors of aspartate- and alanine-aminotransferases with K_i ranging from nanomolar to micromolar values. The half-life of the inhibited complexes varied from 8 min (AspAT-2-AOEP) to 11 h (AspAT-AOMP). Kinetic analysis of the interaction of both enzymes with AOMP suggested the formation of an E–I complex in a single slow binding process. In the case of other compounds, attempt to discriminate between a single- or a double-step mechanism, consistent with an E–I intermediate followed by a slow E–I to E–I* isomerisation process could not be clearly resolved. Spectral studies of the complex formed between PLP-bound enzyme and the aminooxy compound resulted in a shift from 362 nm, the absorption maximum of the native enzyme, to 380 nm, characteristic of the oxime produced. The kinetic parameters for aminooxyphosphonates were compared to those for their carboxylic and aminophosphonic analogues.

KEY WORDS: Aminooxyphosphonates; enzyme kinetics; slow binding inhibition; aspartate-aminotransferase; alanine-aminotransferase.

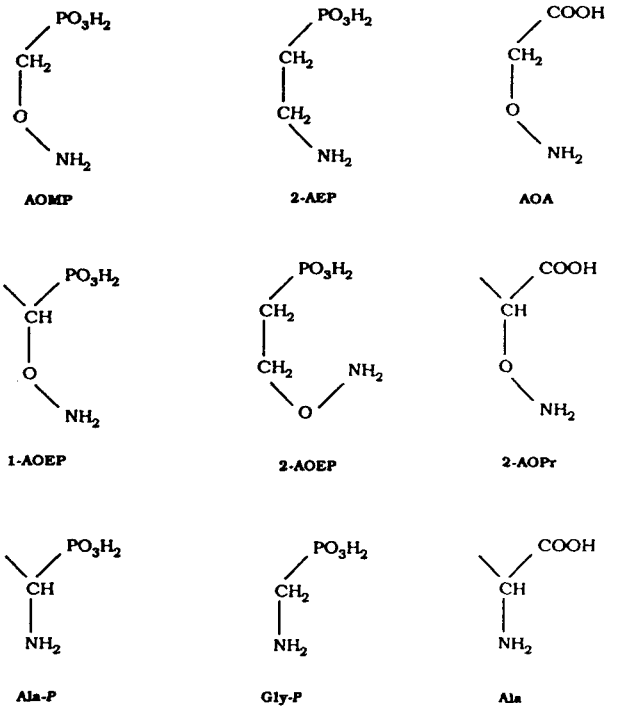
INTRODUCTION

Aspartate aminotransferase (AspAT; EC 2.6.1.1) and alanine aminotransferase (AlaAT; EC 2.6.1.2) are pyridoxal 5′-phosphate (PLP)-dependent enzymes that play a key role in intermediary nitrogen metabolism. They catalyse the reversible transamination of aspartate and 2-oxoglutarate to oxaloacetate and glutamate, and of alanine and 2-oxoglutarate to pyruvate and glutamate, respectively. The cofactor PLP interconverts between an internal aldimine, with Lys-258 in AspAT, and a noncovalently bound pyridoxamine (PMP) form during catalysis[1].

The aminooxy compounds of general formula NH_2-O-R are potent reagents for carbonyl and imine functions[2]. They form oxime complexes with pyridoxal 5′-phosphate (PLP) and with PLP-dependent enzymes as demonstrated with aminooxyacetate[3], the parent compound hydroxylamine[4], L- and D-aminooxysuccinate[5], L- and D-hydrazinosuccinate[6,7] and L-canaline[8]. The nature of the R-chain influences the

* Correspondence.

TABLE 1
Aminooxyphosphonates and related compounds



affinity of the molecule and the stability of the complex formed. If R corresponds to a carbon chain with a terminal carboxylate group, the oximes show a better stability than those formed with carboxylate-free compounds[9]. Hence, it could be expected that replacing the terminal carboxylic group of R by a phosphonic one could provide additional charge stabilization.

Aminooxymethylphosphonic (AOMP), DL-1-aminooxyethylphosphonic (1-AOEP) and 2-aminooxyethylphosphonic (2-AOEP) acids were synthesised and compared to their carboxylic and aminophosphonic analogues as potential inhibitors of cytosolic aspartate and alanine aminotransferases (Table 1).

MATERIALS AND METHODS

The following biochemicals were obtained from Sigma (St Louis, MO): L-alanine, L-aspartic acid, monopotassium α-ketoglutarate, aminooxyacetic acid, NADH, cytoplasmic aspartate aminotransferase (from porcine heart; 360 units per mg of protein)

and alanine aminotransferase (from the same origin; 80 units per mg of protein). Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) were from Boehringer (Mannheim, F.R.G.). All the other chemicals were of analytical grade. IR spectra were recorded with a Specord 75 IR spectrophotometer and ^{1}H-NMR spectra with Testa spectrometers. Enzyme assays and spectral studies were monitored using a Perkin Elmer spectrophotometer.

Synthesis

Aminooxymethylphosphonic Acid (AOMP). AOMP was obtained by a one-pot modification of the literature procedure[10] in the following manner. To a magnetically stirred solution of N-hydroxyphthalimide (10.7 g, 0.065 mol), diethyl hydroxymethylphosphonate[11] (11.0 g, 0.065 mol), and triphenylphosphine (18.2 g, 0.069 mol) in dry tetrahydrofuran (200 ml), diethyl azodicarboxylate (10.9 ml, 0.069 mol) in dry tetrahydrofuran (20 ml) was added dropwise for 40 min while the solution was cooled on an ice-water bath. The mixture was stirred for 12 h at room temperature. Tetrahydrofuran was removed and the residue was treated with dry ether (50 ml). The mixture was left for 3 h and the solid by-products were filtered off. Ether was distilled off from the filtrate and the residue (18.0 g) was dissolved in dry methylene chloride (100 ml). The resulting solution was cooled on an ice-water bath and with stirring, 98% hydrazine monohydrate (3.2 ml, 0.065 mol) was added dropwise for 5 min. Stirring and cooling were maintained for 2 h. Then phthalhydrazide was filtered off and methylene chloride was distilled off to give an oil (13.5 g) which was hydrolyzed with concentrated hydrochloric acid (65 ml) and water (65 ml) for 5 h under reflux. Insoluble materials were then removed by decantation and the hydrolyzate was treated with charcoal. The filtrate was evaporated to dryness and the residue was treated with 96% ethanol (20 ml) and the crude AOMP (6.3 g) removed. Crude AOMP was dissolved in boiling water (250 ml) and the solution after treatment with charcoal was concentrated under atmospheric pressure to half of volume when aminooxymethylphosphonic acid crystallized slowly (5.0 g; 39%), m.p. 189-190°C decomp. (ref.[10] m.p. 189–190°C decomp., ref.[12] 207–208°C decomp.). IR(KBr) : $\tilde{\nu}$ = 670, 920, 945, 1010, 1040, 1080, 1120, 1225, 1520, 2115, 2680, 2920, 3000 cm^{-1}. ^{1}H NMR (100 MHz, D_2O/DCl HMDS) : δ = 4.85 ppm (d, J = 12 Hz, CH_2).

DL-1-Aminooxyethylphosphonic Acid (1-AOEP). 1-AOEP was obtained as des- cribed for AOMP but starting from diethyl DL-1-hydroxyethylphosphonate[13] instead of diethyl hydroxymethylphosphonate. Pure DL-1-aminooxyethylphosphonic acid was obtained after crystallization of the crude compounds (4.9 g) from water (20 ml) to give 3.3 g (38%), m.p. 192–194°C decomp. (ref.[10] m.p. 187–189°C decomp.). IR (KBr) : $\tilde{\nu}$ = 925, 1010, 1040, 1130, 1215, 1575, 2170, 2700, 2940 cm^{-1}. ^{1}H NMR (80 MHz, D_2O/DCl, DSS) : δ = 1.56 (dd, J_{HH} = 7 Hz and J_{HP} = 15 Hz, 3H, $PCCH_3$), 4.56 ppm (m, 1H, PCHC).

2-Aminooxyethylphosphonic Acid (2-AOEP). 2-(Phthaliminooxy)ethyl bromide[14] (2.89 g, 0.0107 mol) and freshly distilled triethyl phosphite (3.0 ml, 0.0175 mol) were

heated gradually to 160°C and then were kept at this temperature for 2 h. The excess of triethyl phosphite was distilled off under reduced pressure and the residue dissolved in dry methylene chloride (20 ml). The resulting solution was cooled on an ice-water bath and, while stirring, 98% hydrazine monohydrate (0.50 ml, 0.010 mol) was added. Stirring and cooling was maintained for 2 h, then phthalhydrazide was filtered off and the filtrate evaporated to remove methylene chloride. The residue was hydrolyzed with concentrated hydrochloric acid (10 ml) and water (10 ml) for 5 h under reflux. The hydrolyzate was treated with charcoal and evaporated to dryness. The residue was treated with 96% ethanol (3 ml) and the crude 2-AOEP (0.50 g) filtered off and crystallized from water (2 ml) and 96% ethanol (1.8 ml) to obtain pure 2-aminooxyethylphosphonic acid (0.40 g; 26%), m.p. 194–196°C decomp. (ref.[12] m.p. 202°C). IR (KBr): $\tilde{\nu}$ = 715, 880, 930, 980, 1005, 1030, 1050, 1095, 1128, 1155, 1260, 1585, 2170, 2690, 2960 cm^{-1}. ^1H NMR (80 MHz, D_2O, DSS): δ = 2.54 (dt, J_{HH} = 7 Hz and J_{HP} = 19 Hz, 2H, CCH_2P), 4.52 ppm (dt, J_{HH} = 7 Hz and J_{HP} = 18 Hz, 2H, $NOCH_2C$).

DL-2-Aminooxypropionic Acid hydrobromide (2-AOPr). 2-AOPr was obtained according to the literature procedure[15].

Enzyme assays

AspAT and AlaAT activities were determined spectrophotometrically by monitoring the formation of oxaloacetic acid from L-Asp and of pyruvic acid from L-Ala with NADH and the coupling enzymes MDH and LDH, respectively. The decrease in absorbance at 340 nm due to the oxidation of the cofactor NADH was followed at 28°C.

The reaction mixture contained 0.1 M Tris-HCl buffer (pH 8), 0.2 mM NADH, 10 mM α-ketoglutarate, 2–20 mM L-Asp or 5–100 mM L-Ala and the enzymes at appropriate concentration. Estimation of the aminotransferase concentration was based on the specific activity and the M_r subunit of AspAT or AlaAT and an extinction coefficient of 6230 M^{-1} cm^{-1} for NADH. Protein content was determined relative to bovine serum albumin using the Biorad-protein Kit.

The inhibitory power of the aminooxy compounds which conforms to a time dependent process was analysed by the onset of inhibition, preincubation studies and reversal of inhibition as described below.

Spectral studies

Before to be submitted to spectral studies, the $(NH_4)_2SO_4$ suspension of AspAT was dialyzed against 1000 volumes of 10 mM Tris-HCl buffer, pH 8 at 4°C for 48 h. A sample was then incubated at 4°C with 20 mM α-ketoglutarate in 0.2 M Tris-HCl buffer pH 8 for 1 h to ensure that the enzyme was converted to the pyridoxal phosphate form and then dialyzed for 24 h against the same buffer[5]. The optical spectrum of a solution of AspAT (7.7 μM) in a volume of 1 ml was recorded between 300 and 500 nm. The aminooxy compound was then added and the spectra were recorded.

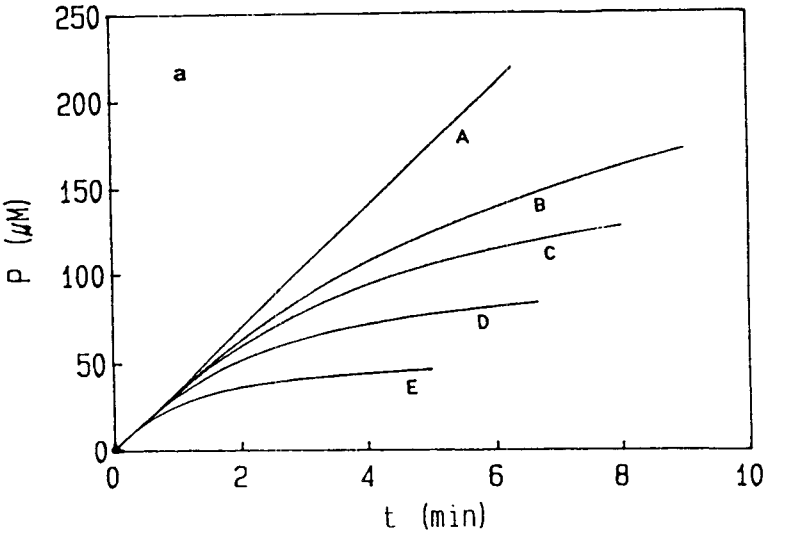


FIGURE 1(a)

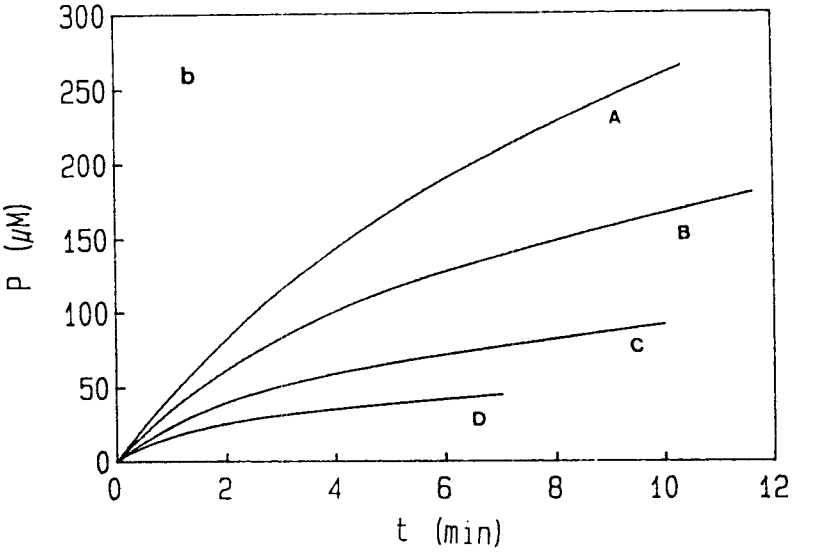


FIGURE 1(b)

FIGURE 1(a,b) Time-dependent inhibition of AspAT by AOMP. (a) The concentration of oxaloacetate produced from aspartate (20 mM) and 2-oxoglutarate (10 mM) was plotted for the following concentrations of AOMP: A, zero; B, 5 μM; C, 7.5 μM; D, 10 μM; E, 20 μM. (b) same as (a), for the following concentrations of aspartate: A, 20 mM; B, 10 mM; C, 5 mM; D, 2.5 mM, at fixed concentration of AOMP (3 μM).

RESULTS AND DISCUSSION

Onset of inhibition of AspAT and AlaAT by aminooxyalkylphosphonates

AOMP, 1-AOEP and 2-AOEP were found to be slow binding inhibitors of AspAT and AlaAT. Progress curves of reactions started by the addition of the enzyme to assay mixtures containing substrate and aminooxy compound showed an initial non-linear decrease in absorbance with time followed by a linear final steady state velocity. The overall activity decreased with inhibitor concentration.

Figure 1 shows the progress curves obtained with AspAT, (a) at a fixed concentration of Asp (4 K_m) and several concentrations of AOMP and (b) at varying concentrations of the substrate (2–20 mM) and fixed concentration of AOMP (3 μM). The curves for all the compounds were analysed by direct fits to an integrated rate equation (1) using a computer program

$$P = v_s t + (v_o - v_s)\,(1 - e^{-k_{obs}t})/k_{obs} \qquad (1)$$

where v_s and v_o are the steady state and initial velocities, respectively, and k_{obs} is the apparent first order rate constant for the approach to steady state. The data fitted to equation (1) allow us to obtain values for K_i and the slow association rate constant. Inhibition was competitive in nature as increasing the amount of the substrate at a fixed concentration of inhibitor resulted in a decrease of k_{obs} obtained from each progress curve (results not shown).

For slow binding inhibitors, Cha[16] and Morrison and Walsh[17] have described two classical mechanisms:

Mechanism A

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E - I$$

In this one step process the rate of association with inhibitor is very much slower than that with substrate at all [S] and [I] values used.

Mechanism B

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E - I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E - I^*$$

There is an initial rapid interaction between enzyme and inhibitor to form an E–I complex which undergoes a slow isomerisation to E–I*; this second step is the rate-limiting step of the reaction.

These two mechanisms can be differentiated by the relationships between k_{obs}, [I] and [S] according to the equations

$$k_{obs} = k_2 + k_1\,[I]/(1 + [S]/K_m) \qquad (A)$$

$$k_{obs} = k_4 + k_3\,[I]/([I] + K_i') \qquad (B) \qquad \text{where } K_i' = K_i(1 + [S]/K_m)$$

TABLE 2
Kinetic parameters for slow binding inhibition of AspAT and AlaAT by aminooxyphosphonates and related compounds

Inhibitor	AspAT				AlaAT			
	K_i (M)	k_{on} ($M^{-1}s^{-1}$)	k_{rgn} (s^{-1})	$t_{1/2}$ (h)	K_i (M)	k_{on} ($M^{-1}s^{-1}$)	k_{rgn} (s^{-1})	$t_{1/2}$ (h)
AOMP	6.9×10^{-9} (9.4×10^{-9})	2.4×10^{3}	1.7×10^{-5}	11	6.3×10^{-9} (7.1×10^{-9})	9.5×10^{3}	6.0×10^{-5}	3.2
AOA	10.5×10^{-8}	5.2×10^{2}	5.5×10^{-5}	3.5	4.0×10^{-8}	5.0×10^{3}	2.0×10^{-4}	0.96
AOA[a]	9.5×10^{-8}	4.2×10^{2}	4.0×10^{-5}					
1-AOEP	2×10^{-7} (3.2×10^{-7})	8.7×10^{2}	1.8×10^{-4}	1	1.45×10^{-6}	4.6×10^{2}	6.66×10^{-4}	0.25
2-AOPr	14×10^{-8} (12.6×10^{-8})	4.1×10^{2}	5.7×10^{-5}	4.8	10×10^{-8} (5.6×10^{-8})	9.2×10^{2}	9.2×10^{-5}	2.1
2-AOEP	2.6×10^{-6}	5.4×10^{2}	1.4×10^{-3}	0.133	42×10^{-9} (52×10^{-9})	5.0×10^{3}	2.1×10^{-4}	0.92

[a]: values found in the literature[3]

Hence, our data were analysed following classical criteria[16,17]. These criteria, quoting as an example AspAT-AOMP, apply to all the other complexes except AspAT-2-AOEP and AlaAT-1-AOEP which are particular cases. (a) The initial velocity is independent of [I] for mechanism A but varies as a function of [I] for mechanism B due to the rapid initial formation of the intermediate E–I complex. The progress curves depicted in Fig. 1a for AspAT-AOMP show that initial velocity cannot be differentiated from the control velocity; moreover, the double reciprocal plot of initial velocity and substrate concentration obtained from Fig. 1b leads to calculation of a constant value close to the enzyme K_m. Both data support mechanism A. (b) The apparent first order rate constant k_{obs} varies as a linear function of [I] which again favours mechanism A, since an hyperbola should account for mechanism B. The bimolecular association rate constant k_{on}, calculated from the slope of the line was found to be 2.4×10^3 $M^{-1}s^{-1}$ for the complex AspAT-AOMP and varied between 4×10^2 and 9×10^3 $M^{-1}s^{-1}$ for the other complexes with both enzymes (Table 2). These values, much slower than diffusion control ($k_1 \sim 10^7$, 10^8 $M^{-1}s^{-1}$), may imply some set of energy barriers that must be surmounted to align the inhibitor and active site during binding. (c) This criterion requires also the measurement of the dissociation rate constant, k_{rgn} (see Table 2 and *Reversal of inhibition*). The plot of $1/(k_{obs} - k_{rgn})$ *versus* 1/[I] passes through the origin for AspAT-AOMP and AlaAT-AOMP, which predicts mechanism A; it gives an intercept near the origin for the other complexes, which may be consistent with the predicted abscissa intercept $(-1/K_i')$ for mechanism B if the initial inhibition constant is large ($K_i \gg$ [I]).

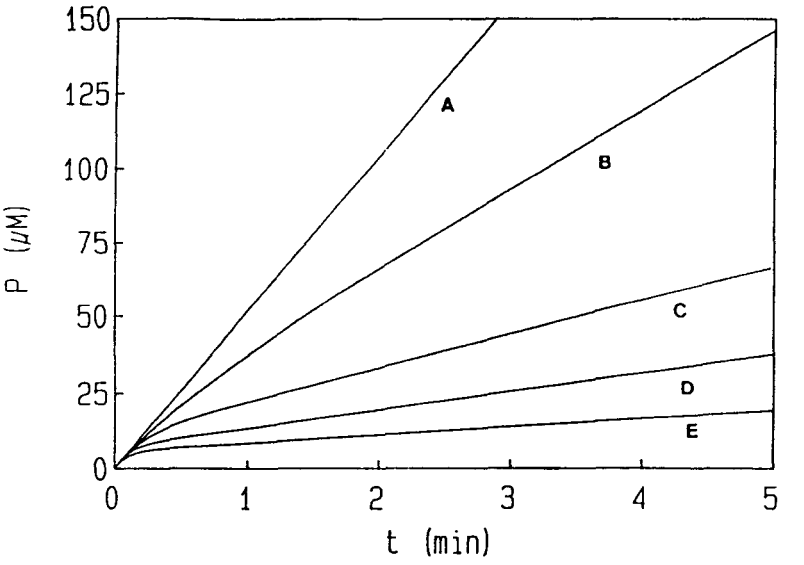


FIGURE 2 Time-dependent inhibition of AlaAT by 1-AOEP at 50 mM alanine and 20 mM 2-oxoglutarate for the following concentrations of 1-AOEP: A, zero; B, 6 μM; C, 20 μM; D, 50 μM; E, 100 μM.

A particular case of mechanism B[17] was found for AspAT-2-AOEP and AlaAT-1-AOEP. The progress curves of Figure 2 show that the change in going from the initial to the final steady state rate was rapid indicating that the forward and reverse isomerisation rates associated with mechanism B were relatively high. Analysis of the final velocity of these curves by a Dixon[18] plot yielded the value for the overall inhibition constant K_i^* : 2.6×10^{-6} M and 1.4×10^{-6} M for the complexes AspAT-2-AOEP and AlaAT-1-AOEP, respectively. However, confirmation of two-stage interaction between enzyme and inhibitor would require the use of rapid reaction techniques.

Preincubation studies

The overall inhibition constants K_i^* of AspAT and AlaAT for AOMP, 1-AOEP, 2-AOEP were also determined, as an alternative to progress curve analysis, by preincubating enzyme and inhibitor until an equilibrium was reached (1 h). The residual enzyme activity was then determined upon addition of substrates. The variation in the final steady-state velocity with inhibitor concentration was described by equation (2) for classical linear competitive inhibition

$$v_s = V_{\max}[S]/\,[K_m(1 + [I]/K_i^*) + [S]] \qquad (2)$$

where [S] is the concentration of substrate, [I] is the inhibitor concentration and K_i^* is the overall inhibition constant.

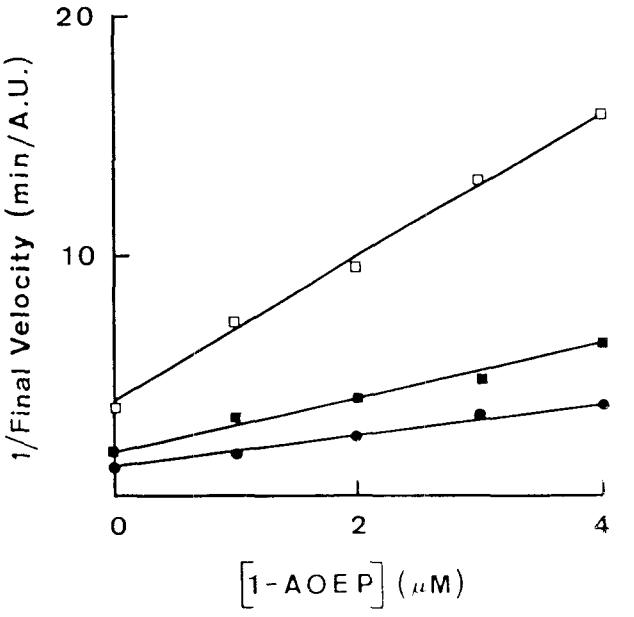


FIGURE 3 Linear regression of 1-AOEP against the reciprocal of the final steady state velocity obtained from preincubation mixtures (enzyme, inhibitor, buffer) at three AspAT concentrations: 0.5 nM (□), 1.0 nM (■), 1.5 nM (●). An average K_i of 0.32 μM was calculated from the slope of the plots.

By plotting $1/v_s$ *versus* [I] a slope equal to $K_m/([S] \times V_{max} \times K_i^*)$ allowed the determination of K_i^* (Figure 3). These values, shown in parentheses in Table 2, were close to the values obtained from the progress curves analysis.

Reversal of inhibition

The rate of recovery of catalytic activity of the fully inhibited enzyme was monitored spectrophotometrically: as an example, AlaAT (3 μM) was inactivated 2 h at 20°C in 200 mM Tris-HCl buffer pH 8 in the presence of AOMP (120 μM). A control sample containing enzyme and buffer was kept 3 h at 20°C; enzyme activity determined at time 0, 1, 2 and 3 h showed the stability of the protein. A sample of 5 μl was then diluted into 1 ml of the standard assay mixture containing 50 mM L-Ala and the reaction was recorded. From the progress curve (Figure 4) we have determined the pseudo first order rate constant for the regain of enzyme activity, k_{rgn}, by plotting $\ln[100(1-v/v_s)]$ *versus* time where v is velocity at time t and v_s is the theoretical final velocity reached (control velocity). A value of k_{rgn} of 6.0×10^{-5} s^{-1} corresponding to a half life of 3.2 h was determined for AlaAT-AOMP from the slope of the plot (Figure 4, inset); for the complex AspAT-AOMP the reversal of inhibition was even slower ($t_{1/2}$ = 11 h). The higher dissociation rate constants were found to be 1.4×10^{-3} s^{-1} ($t_{1/2}$ = 8 min) and 6.66×10^{-4} s^{-1} ($t_{1/2}$ = 15 min) for AspAT-2-AOEP and AlaAT-1-AOEP, respectively.

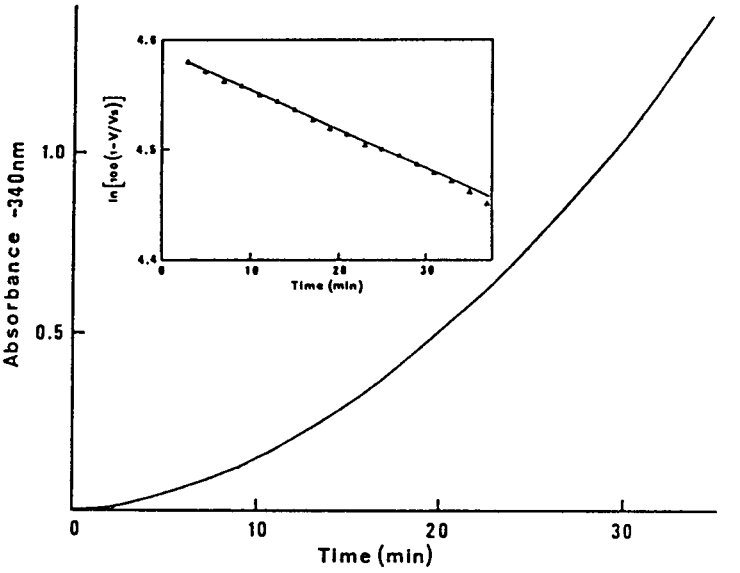


FIGURE 4 Slow reversal of inhibition of AlaAT from AOMP complex after 2 h incubation. *Inset*, k_{rgn} was calculated from the slope of the line.

These values of k_{rgn} are one or two order of magnitude higher than those determined for the other compounds tested (Table 2) and are close to the value reported[5] for AspAT-L-aminooxysuccinate (1.5×10^{-3} s^{-1}).

Spectral studies

Spectral changes of AspAT (7.7 μM) upon successive additions of AOMP (5 μM) were recorded at pH 8 between 500 and 300 nm. The PLP spectrum maximum at 362 nm, reflecting the internal aldimine between the coenzyme and Lys-258 was shifted to a longer wavelength, 380 nm, that is characteristic of an oxime formation between PLP and an aminooxy compound (Figure 5).

Comparison of the properties of aminooxyphosphonates with related compounds

(a) Effect of aminomethyl- ('Gly'-P), 1-aminoethyl- ('Ala'-P), and 2-aminoethylphosphonic (2-AEP) acids. These aminophosphonates did not exhibit inhibitory properties towards AspAT and AlaAT when added at the concentration of 10 mM in the assay mixture containing 5 mM of substrate. Therefore, the replacement of the aminooxy function by an amino group or an amino methylene group (compare AOMP, 'Gly'-*P* and 2-AEP) abolished the inhibitory properties of these molecules. It is noteworthy that 'Ala'-*P*, the phosphonic analogue of Ala was found to be a potent slow binding inhibitor with a long-lived E–I* complex ($t_{1/2} \sim 25$ days) of an other PLP-requiring enzyme, alanine racemase[19] whereas 1-AOEP was a weak inhibitor of alanine racemase from *Pseudomonas aeruginosa* (results not shown).

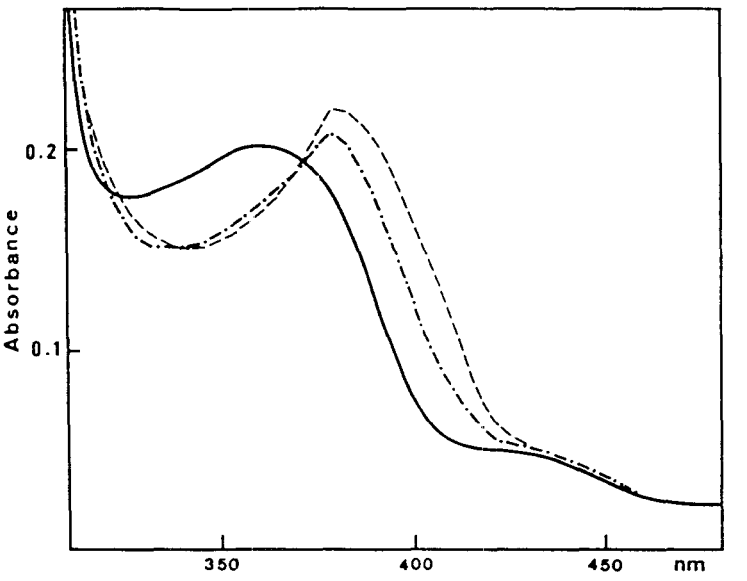


FIGURE 5 Spectral changes of AspAT before and after addition of AOMP. The spectra of the PLP form of the enzyme (7.7 μM) (——), and after addition of 5 μM (—·—·—) and 15 μM (— — —) inhibitor. Another addition up to 30 μM did not modify the equilibrium.

(b) Effect of aminooxyacetic (AOA) and DL-2-aminooxypropionic (2-AOPr) acids. The data illustrated in Table 2 show that AOMP is about ten-fold more potent than AOA in inhibiting the two aminotransferases whereas 2-AOPr is a better inhibitor than 1-AOEP. In the former case the phosphonic group might induce a better stabilization of the complex ($t_{1/2}$ for activity regain, 11h) and in the latter case the orientation of the carboxylic group may favour a better interaction with a positively charged residue. At pH 8, there is no difference in the overall charge of these molecules, but the size and shape of the tetrahedral $-PO_3H^-$ group, as opposed to the planar $-COO^-$ group, may differentiate the behaviour of these compounds according to the topology of the active site. The same behaviour of the two aminotransferases towards the two series of compounds fits in with spatial structure similarity and invariant residues aligned around the PLP binding site of the two enzymes[20].

The rate constants for the progress of AOA and 2-AOPr inhibition are near those found for AOMP, 1-AOEP and 2-AOEP with AspAT but markedly smaller than the second order rate constant reported[5] for L-aminooxysuccinate (1×10^4 M^{-1} s^{-1}) and L-hydrazinosuccinate inhibition (7.4×10^4 M^{-1} s^{-1}). These two molecules are close analogues of the substrate L-Asp; the distal and proximal –COOH groups can serve as recognition points for ion pair formation with Arg-292 and Arg-386. An E–I complex is rapidly formed, supporting mechanism B.

In conclusion, the aminooxyphosphonates synthesised here are new potent time-dependent inhibitors of PLP-dependent aminotransferases. Except for AOMP, no clear-cute distinction between mechanism A or B was demonstrated for the other

aminooxyphosphonates. 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 complex (a special case of mechanism B) to a tightened E–I* complex. The same ambiguity in the interpretation of the results was reported for the time dependent inactivation of 1-aminocyclopropanecarboxylate deaminase by 1-aminocyclopropanephosphonate[21].

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