THE SYNTHESIS AND PROPERTIES OF PHOSPHOPYRIDOXYL AMINO ACIDS

E.S. Severin, N.N.Gulyaev, E.N.Khurs and R.M.Khomutov

Institute of Molecular Biology, Academy of Sciences of USSR, Moscow

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The conversion of substrate into product catalyzed by an enzyme with a prosthetic group is known to involve a number of stages during which the substrate is bound covalently with the coenzyme. For pyridoxal enzymes, and particularly for transaminases, Schiff bases formed by amino acids and pyridoxal-5-phosphate (PLP) are considered the major type of these intermediate complexes. From a purely chemical viewpoint it is evident, that besides these major forms there must also exist others, e.g., containing no double bond between coenzyme and substrate.

We have recently proposed, that compounds which are structural analogues of the substrate-coenzyme complexes may be inhibitors of prosthetic group-containing enzymes, particularly, of the PLP-enzymes /1/. In the present paper we report the synthesis of previously unknown N-(pyridoxyl-5'-phosphate)-amino acids (P-Pyl-amino acids) and the results of the studies on their interaction with some transaminases whose coenzyme is PLP and whose substrates are L-amino acids.

MATERIALS AND METHODS

The Synthesis of P-Pyl-Amino acids. A methanolic solution of dipotassium pyridoxal-5-phosphate (1 mmole) was added in small portions to 1 mmole of the potassium salt of the amino acid in dry methanol (total volume 15 ml). In 30 min the solution obtained was treated at 5° with 0.02 M $NaBH_4$ (purity 50%) (added in portions), the mixture was filtered, acidified with acetic acid

and evaporated. The precipitate was dissolved in water and chromatographed on ion exchange resin, Amberlite XE-64 (2.5x40 cm column), with water as the eluting solvent.

Fractions absorbing at 330 mμ were collected and freeze-dried. The dry residue was crystallized from ethanolic ammonia and purified additionally by preparative electrophoresis (30 v/cm; buffer: pyridine-acetic acid-water, 1:5:94; Whatman N^{o} 3MM; spot location - by luminescence in the UV or by Gibbs reaction /2/). The data on elementary analysis and the R_f-values of the compounds are presented in Table 1.

Enzymes, test for activity and extent of inhibition.

Glutamate-aspartate transaminase from pig hearts (GOT, E.C. 2.6.1.1), pyridoxal form, was isolated by method /3/, its activity and the extent of inhibition were determined by method /4/, GOT, apo-enzyme, was obtained by method /5/; its activity was tested as follows. 0,02 ml apo-enzyme solution (0,02-0,04 mg of protein) was incubated during 10 min at 37^{o} with 2 ml of 0,025 M phosphate buffer, pH 7,4. Subsequently 0,02 ml of 0,025 M phosphate buffer, pH 7,4, containing 8.10^{-8} mole of PLP, was added and the incubation at 37^{o} was continued for 3 min. The following treatment was performed as described /4/ for the pyridoxal form of GOT.

Estimation of the extent of inhibition was performed in the same manner as the test for activity, except that 2 ml of buffered inhibitor solution was taken instead of 2 ml of buffer. The times of the preincubation with inhibitor were 10 min, 30 min, 1 h and 2 h. The times of the incubation with PLP were 3 min, 10 min and 30 min. The initial rates (15-45 sec) of the enzymatic reactions in the presence and in the absence of inhibitors were compared.

GOT, pyridoxamine form, was obtained by method /6/. The activity and the extent of inhibition were estimated in the same manner as those of the pyridoxal form of GOT /4/.

Alanine-glutamate transaminase (E.C. 2.6.1.2) was obtained from pig heart muscle /7/; its activity and the extent of the inhibition were determined as described in ref. /7/.

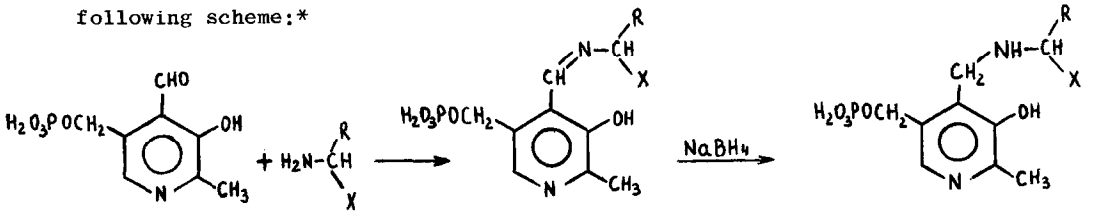
Tyrosine (phenylalanine)-glutamate transaminase (E.C. 2.6.1.5) was obtained from adult rats brain in a partially purified form as described in ref. /8/. The determination of its activity and of the extent of inhibition was performed as described in ref. /8/.

γ-Aminobutyrate transaminase. 10% suspension of rat brain acetone powder /9/ was used as enzyme preparation; the test for the activity was performed as described in ref. /9/, and the extent of the inhibition estimated as described in ref. /9/.

RESULTS AND DISCUSSION

Previously described has been the synthesis of some N-pyridoxyl-amino acids via condensation of pyridoxal with amino acids in dry methanol and subsequent catalytic hydrogenation of the intermediate N-pyridoxylidene derivatives /10/. It was anticipated that the same approach could be applied to the synthesis of P-Pyl-amino acids, but after Fisher's publication /11/, it seemed reasonable to apply for the purpose sodium borohydride as a more selective reducing reagent.

A number of P-Pyl-amino acids have been synthesized according to the following scheme:*



I R=CH_3, x=COOH; II R=$-CH_2COOH$, x=COOH; III R=$-(CH_2)_2COOH$, x=COOH; IV R=$-CH_2C_6H_5$, x=COOH; V R=p-HO-$C_6H_4CH_2-$, x=COOH; VI R=$-(CH_2)_2COOH$, x=H

* Our method has been applied by Tolosa et al. /12/ to the synthesis of N,N'-dipyridoxylphosphate cystine. During the preparation of this paper, a communication by M.Ikawa (Ikawa,M.,Arch.Biochem.Biophys.,118, 497, 1967) appeared concerned with the synthesis of compounds I,II and V by a somewhat different method.

The compounds obtained were characterized by mobility in paper electrophoresis (the conditions similar to those of the preparative runs, but the time of the run was 15 min, the paper was Whatman 1M and the potential gradient 80 $\frac{V}{cm}$) and chromatography (Table 1).

All of them exhibit characteristic absorption maxima at ca. 330 mμ (Fig. 1).

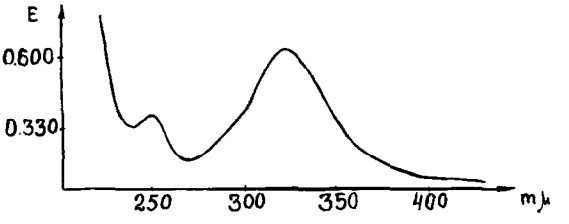


Fig. 1 Typical UV-spectrum of P-Pyl-amino acid. P-Pyl-L-glutamic acid ($C = 10^{-4}M$) in phosphate buffer (pH 7.2)

Table 1

Properties of P-Pyl-amino acids

P-Pyl-	[α]$_{365}$ *)	R_f **)	Formula	Analyses, % C Calc.	C Found	H Calc.	H Found	N Calc	N Found	P Calc	P Fou
Alanine (I)	+11,2°	0,6	$C_{11}H_{17}N_2O_7P$	41,25	39,84	5,33	5,16	8,74	8,55	9,67	10,
Aspartic acid (II)	+27,0°	0,5	$C_{12}H_{17}N_2O_9P$	39,56	39,27	4,70	4,82	7,69	7,99	8,51	8,
Glutamic acid (III)	+25,0°	0,5	$C_{13}H_{19}N_2O_9P$	41,27	39,90	5,06	5,29	7,43	7,17	8,19	8.
Phenyl-alanine (IV)	-31,7°	0,7	$C_{17}H_{21}N_2O_7P$	51,52	50,96	5,34	5,42	7,07	6,75	7,82	8
Tyrosine (V)	-74,4°	0,65	$C_{17}H_{21}N_2O_8P$	49,52	49,13	5,13	5,28	6,79	6,24	7,51	7
γ-Amino-butyric acid (VI)		0,56	$C_{12}H_{19}N_2O_7P$	43,12	42,81	5,76	5,66	8,38	8,70	9,27	9

P-Pyl-amino acids are stable during storage, except the compound III which durir several weeks of storage was converted into a more acidic substance as revealed by paper electrophoresis, most probably this was P-Pyl-pyroglutamic acid.

*) Optical rotations determined with a photoelectric polarimeter with 0,8% solutions (in 0,1 N HCl) at temperatures 25-28°.

**) Descending chromatography, paper Whatman N° 1M, ethyl acetate-pyridine - water (2:1:2), detection by Gibbs reagent /2/.

First of all we investigated the interaction of P-Pyl-glutamic acid (III) with the aldehyde form of GOT, which was inhibited by III but to a small extent after 10 min preincubation (14%, $c = 10^{-3}$M). However, after 60 min preincubation the extent of the inhibition increased up to about 25%.

Transaminases of tyrosine and phenylalanine, of γ-aminobutyric acid and of alanine were inhibited, respectively, by compounds V(26%, c = 2,5 10^{-4}M), IV (29%, c = 2,5 10^{-4}M), VI (30%, c = 10^{-3}M) and I (40%, c = 10^{-3}M).

If it were the pyridine moiety acceptor site of transaminases that is responsible for the binding of the inhibitors, a much stronger effect of P-Pyl-amine acids upon apo-GOT compared with that upon the aldehyde form of enzyme could be anticipated. In accord with this, apo-GOT was inhibited by much lower concentrations of the compounds (Table II); as expected, the compounds III and II which are close structural analogues of the substrates of this enzyme, exhibited the greatest activity.

Table II

The inhibition of apo-GOT by P-Pyl-amino acids in phosphate buffer, pH 7,4

M Concentration / P-Pyl-	10^{-3}	10^{-4}	10^{-5}
	% of inhibition after 10 min incubation with apo-enzyme		
alanine (I)	80	23	0
aspartic acid (II)	60	37	12
glutamic acid (III)	72	45	27
phenylalanine (IV)	-	8	0
tyrosine (V)	-	20	0
γ-aminobutyric acid (VI)	58	20	0

The amino form of GOT was inhibited by compounds II and III (c= 10^{-5}M) to the same extent as apo-GOT (16 and 26%, respectively). Most probably, this is explained by the relatively diss tion into apo-enzyme and pyridoxaminephosphate in diluted solution. Hence, P-Pyl-amino acids, though not very efficient, are inhibitors of PLP-enzymes (Table II). This observation would be certainly of a marginal importance if

the former were simply reversible inhibitors. Surprisingly enough, this was not the case, since the extent of inhibition of the pyridoxal form of GOT depended on the time of preincubation. Moreover, apo-GOT inhibited with compound III ($c=10^{-3}$M, 10 min preincubation) did not recover its activity after prolonged incubation with PLP (up to 30 min). Similarly, the inhibition of apo-GOT with compound III ($c=10^{-4}$M) also increased with the time of preincubation (10 min - 45%, 30 min - 62%, 1 h - 78%, 2 h - 94%). These facts suggested that the inhibition is irreversible and probably involves the formation of covalent bonds between enzyme and inhibitor. This possibility seems interesting and is the subject of the following studies /13/.

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