N-(5′-PHOSPHO-4′-PYRIDOXYL)AMINES AS SUBSTRATES FOR PYRIDOXINE (PYRIDOXAMINE) 5′-PHOSPHATE OXIDASE[1]

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A preparation of pyridoxine (pyridoxamine) 5′-phosphate oxidase, with a specific activity of 9,400 nmoles/hr/mg protein, 10-fold higher than that previously reported, was used to study the oxidation of various N-(5′-phospho-4′-pyridoxyl)amines. Values for K_m, from 3.1×10^{-5} M to 1.6×10^{-3} M, and for V_{max}, relative to pyridoxamine-P, of 20 to 140% were obtained. Compounds lacking a 5′-phosphate were not substrates, and the enzymic reaction was dependent on the presence of both FMN and O_2. N-(phosphopyridoxyl)-L-amino acids had lower K_m's than the corresponding -D-amino acid compounds. When 1-^{14}C-N-(phosphopyridoxyl)glycine was used as a substrate, no $^{14}CO_2$ was evolved, and 1-^{14}C-glycine was detected in the incubation mixture.

Pyridoxine-P (pyridoxamine-P) oxidoreductase (deaminating, EC 1.4.3.5) was first partially purified from rabbit liver by Pogell (1) and later studied by Wada and Snell (2). Using a 184-fold purified preparation, Korytnyk et al. have studied the properties of substrates modified in the 5′-position (3) and have found that the 4-vinyl analog is the most potent inhibitor of the oxidase yet studied (4,5). The main results from these earlier studies are: a dianionic phosphate in the 5′-position is required for substrate activity; both pyridoxine- and pyridoxamine-5′-phosphates are active as substrates; analogs having a π-system in the 4′-position are good inhibitors, viz. pyridoxal-P, pyridoxal-P oxime, and 4-vinylpyridoxine-P; the oxidase is dependent upon FMN; O_2 is required for the enzymic reaction. The oxidase reaction has also been postulated to be a key control reaction in vitamin B_6 metabolism (6).

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N-(4′-pyridoxyl)amino acids were first synthesized by Heyl et al. (7) and were found to have low vitamin B_6 activity in rats (7) and microorganisms (8). Ikawa extended this work to the synthesis of some of the N-(5′-phospho-4′-pyridoxyl)amino acids (9).

The present work describes an improved, partial purification of pyridoxine-P oxidase. Using this preparation and various N-(phosphopyridoxyl)amines synthesized by methods previously developed (7,9), the effect of modification at the 4′-position on substrate activity has been examined.

MATERIALS AND METHODS

Purification of Pyridoxine-P Oxidase. Rabbit liver was held at -20° until used. Diced liver (150 g) was homogenized for 5 min at low speed in a Waring blendor with 600 mℓ of 0.02 M KP_i (pH 7.0) containing 10^{-4} M $HSCH_2CH_2OH$. The suspension was centrifuged for 30 min at 18,000 x g and the precipitate discarded. The pooled supernatants from two such extractions were adjusted to pH 4.5 with 2 N acetic acid, and the precipitate removed by centrifugation. The supernatant was adjusted to pH 5.0 with 10% KOH and to 0.12 M with solid KCl. Absolute EtOH (0.3 vol at -20°) was added dropwise in a -20° room. The precipitate, collected by centrifugation at -10° (10 min at 10,000 x g), was suspended in 0.05 M KP_i (pH 8.0). Insoluble material was removed by centrifugation. The clear yellow solution was poured over a DEAE-Sephadex A-50 column (5 x 43 cm), equilibrated with 0.05 M KP_i (pH 8.0), and material eluted with a linear gradient established between 1 ℓ of 0.05 M KP_i and 1 ℓ of 0.2 M KP_i, pH 8.0. Fractions containing oxidase activity were pooled and brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected and dialyzed overnight against 0.02 M KP_i (pH 7.0). The dialysate was chromatographed on a Sephadex G-100 column (2.5 x 97 cm). Active fractions were pooled and poured over a column of calcium phosphate gel (0.9 x 10 cm) prepared according to Massey (10) and equilibrated with 0.02 M KP_i (pH 7.0). Material was eluted with a 100-mℓ linear gradient of 0.02 to 0.1 M KP_i (pH 7.0). Fractions con-

TABLE I

Summary of Purification of Pyridoxine-P Oxidase*

Fraction	Volume	Protein	Specific activity	Total activity	Yield	Purification factor
	mℓ	mg/mℓ	unit/mg	units	%	
Crude extract	1205	32.8	16.9	670,000	100	1
Acid supernatant	1095	20.2	26.7	592,000	88.5	1.6
EtOH precipitate	127	31.8	105	425,000	63.4	6.2
DEAE fraction	15.8	20.2	602	190,000	28.7	35.6
G-100 fraction	42	1.30	2350	132,300	19.5	138
$CaPO_4$ gel fraction	9	0.39	9390	32,600	4.9	556

*Specific activities of oxidase preparations are given as nmoles of pyridoxal-P formed/hr/mg protein.

taining the highest specific activity were pooled. As summarized in Table I, pyridoxine-P oxidase was purified 556-fold in 4.9% yield by this procedure.

Assays. Pyridoxine-P oxidase was assayed according to Wada and Snell (2) in 0.2 M Tris-HCl (pH 8.0) using pyridoxamine-P as substrate, except that 2 nmoles FMN and 50 µg bovine serum albumin were routinely included when oxidase preparations with specific activities >1000 were used. One enzyme unit catalyzes the formation of 1 nmole pyridoxal-P per hr, measured spectrally as the hydrazone. Protein was determined by the method of Lowry et al. (11).

Anaerobic reactions were run in single-sidearm Thunberg tubes, which had been evacuated and flushed three times with vandate-washed (O_2-free) N_2.

Synthesis of N-(5'-phospho-4'-pyridoxyl)amines. The general method has been described previously (7,9). The synthesized N-(phosphopyridoxyl)amines (Table II) were free of both pyridoxal- and pyridoxamine-P. 1-^{14}C-N-(phosphopyridoxyl)glycine was synthesized as follows: 50 µCi of 1-^{14}C-glycine, from International Chemical & Nuclear (11.5 mCi/mmole in 0.01 N HCl) was diluted with unlabeled glycine to give 40 µmoles in 0.2 mℓ. Added to this were pyridoxal-P (40 µmoles), 0.01 mℓ of 42.5% KOH, and a few mg of PtO_2; reduction was effected by bubbling H_2 through the solution. The solution containing the product was removed from the catalyst by pipet and neutralized with 0.005 mℓ of

TABLE II

K_m and V_{max} Values for N-(phosphopyridoxyl)amines*

N-(5′-phospho-4′-pyridoxyl)-amine	Apparent K_m	Relative V_{max}†
	$\times 10^5$ M^{-1}	%
Pyridoxamine-P‡	1.3	100
Glycine	5.2	110
β-Alanine	11	85
L-Alanine§	22	140
D-Alanine	77	130
L-α-Aminobutyrate	9.1	96
D-α-Aminobutyrate	29	120
α-Aminobutyrate	77	130
L-Serine§	13	39
L-Leucine	7.5	86
D-Leucine	12.5	120
Benzylamine	3.3	120
L-Phenylalanine	9.5	54
L-Tyrosine§	3.1	57
D-Tyrosine	160	55
L-Tryptophan§	12	18

*K_m and V_{max} values were determined from Lineweaver-Burk plots.

†Given as V_{max}/V_{max} for pyridoxamine-P $\times$ 100.

‡This parent amine is shown for comparison.

§Previously synthesized (9).

concentrated HCl. The 1-^{14}C-N-(phosphopyridoxyl)glycine, which contained a minor amount (<2%) of unreacted 1-^{14}C-glycine and some dephosphorylated product, was used without further treatment.

RESULTS AND DISCUSSION

Table II lists the N-(phosphopyridoxyl)amines synthesized in this study, together with K_m and relative V_{max} values. All the N-(phosphopyridoxyl)amines are substrates of pyridoxine-P oxidase, but they vary considerably in effectiveness. Those with aromatic substituents (except benzylamine) appear to have slower V_{max}'s than those with aliphatic substituents (except serine). However, for both classes, the K_m values vary over a considerable range. The most striking relationship between structure and substrate effectiveness is illustrated by the differences in the K_m values for various D,L pairs. As most strikingly shown by the tyrosine compounds with a 50-fold difference in K_m, the N-(phos-

phopyridoxyl)D-amino acids are much less effective substrates than the corresponding L-compounds. If N-(pyridoxyl)amino acids can be phosphorylated *in vivo*, these observations provide a possible explanation of the vitamin B_6 activity of these compounds (7,8).

Wada and Snell (2) reported that pyridoxine-P oxidase slowly oxidized pyridoxamine at pH 10. Using the 556-fold-purified oxidase, we could detect no conversion of pyridoxamine, N-(pyridoxyl)glycine, -β-alanine, or -L-tyrosine to pyridoxal, even at pH 10 and 10 mM substrate concentrations. This result suggests that a 5'-phosphate moiety is absolutely required for activity.

Pogell (1) demonstrated an oxygen dependence for the enzymic formation of pyridoxal-P from pyridoxamine-P. This same dependence exists when N-(phosphopyridoxyl)amines are used as substrates. When the reactions were run anaerobically, <6% (4.3-5.7) of the control activity was observed. The oxidation of these secondary amines is also dependent on FMN. Activity can be abolished by acid:$(NH_4)_2SO_4$ precipitation (12) and reconstituted only by the addition of FMN; FAD and riboflavin are both inactive as coenzymes, even at 10^{-5} M. Finally, if mixtures of pyridoxamine-P and an N-(phosphopyridoxyl)amine are used as substrate in incubations with the oxidase, the formation of pyridoxal-P is not additive. These results indicate that the same enzymic activity is responsible for the oxidation of both pyridoxamine and N-(phosphopyridoxyl)amines.

In an attempt to determine the amine products of the enzymic oxidation of N-(phosphopyridoxyl)amines, 1-^{14}C-N-(phosphopyridoxyl)glycine was synthesized and used as substrate. Several experiments were performed in 0.1 M Tris with varying concentrations of both ^{14}C-substrate and oxidase in final volumes of 0.13 to 0.26 mℓ. The reactions were stopped after 3 hr at 37° by the addition of 100% CCl_3COOH. Under these conditions, up to 25% of the substrate was converted to products. Hydroxide of Hyamine (Packard) was used to trap any evolved $^{14}CO_2$, but none was detected. Aliquots of the incubation mixtures were assayed for pyridoxal-P and applied to Whatman 1 MM paper for overnight development in ascending t-amyl alcohol:acetone:H_2O:CH_3COOH (8:7:4:1, v:v:v:v) (9).

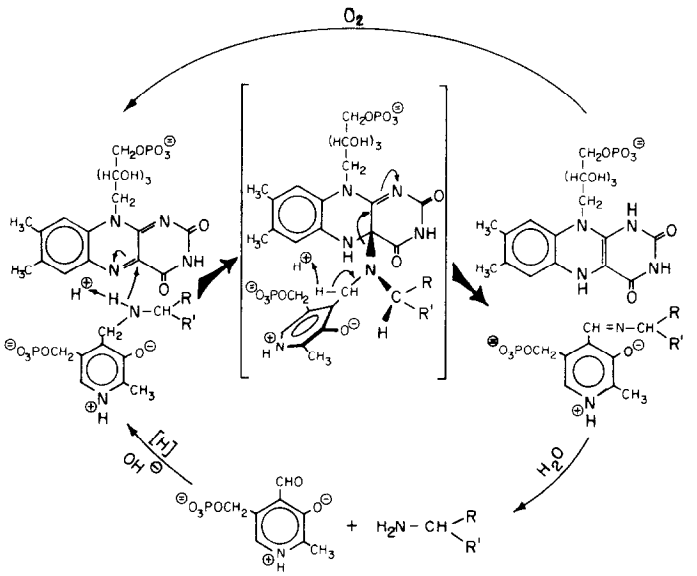

Figure 1. Proposed scheme for the enzymic oxidation of N-(phosphopyridoxyl)amines. R=H, COO^-, and R' as appropriate for the compounds listed in Table II. The supposed covalent intermediate has been drawn to emphasize that the ring systems are nonparallel if the relevant orbitals are aligned so as to facilitate formation of the product.

The increase of ^{14}C-glycine detected (Nuclear-Chicago Actigraph III strip scanner) on the developed chromatograms paralleled the amount of pyridoxal-P formed, and no additional ^{14}C-compounds (other than starting material) could be detected. From this evidence, it can be concluded that ^{14}C-glycine and pyridoxal-P are the products of the enzymic oxidation.

The results of this work are summarized in Figure 1. The lower left-hand reaction is the synthesis of the N-(phosphopyridoxyl)amine substrate. The middle shows the progress of the enzyme reaction based on a generalized mechanism of flavin catalysis proposed by Hamilton (13). As indicated by model studies (14), it is the dipolar B_6 form, the one present at physiological pH, that interacts best with the flavin. It should be noted that in the supposed covalent intermediate, proper alignment of the orbitals for formation of a Schiff's base requires that the flavin and B_6 ring systems lie in nonparallel planes, as indicated. Both the dianionic phosphates are probably required for binding (3,15,16). The basis for the stereochemical differentiation of R and

R', as reflected by differences in K_m values for D and L forms, remains obscure and awaits a more detailed examination of both enzyme structure and enzyme substrate interactions.

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