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EVIDENCE FOR THE REGULATION OF PYRIDOXAL 5′-PHOSPHATE FORMATION IN LIVER BY PYRIDOXAMINE (PYRIDOXINE) 5′-PHOSPHATE OXIDASE[1]

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SUMMARY: Pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5) purified from rabbit liver is competitively inhibited by the reaction product, pyridoxal 5′-phosphate. The K_i, 3 μM, is considerably lower than the K_m for either natural substrate (18 and 24 μM for pyridoxamine 5′-phosphate and 25 and 16 μM for pyridoxine 5′-phosphate in 0.2 M potassium phosphate at pH 8 and 7, respectively). The K_i determined using a 10% rabbit liver homogenate is the same as that for the pure enzyme; hence, product inhibition in vivo is probably not diminished significantly by other cellular components. Similar determinations for a 10% rat liver homogenate also show strong inhibition by pyridoxal 5′-phosphate. Since the reported liver content of free or loosely bound pyridoxal 5′-phosphate is greater than K_i, the oxidase in liver is probably associated with pyridoxal 5′-phosphate. These results also suggest that product inhibition of pyridoxamine-P oxidase may regulate the in vivo rate of pyridoxal 5′-phosphate formation.

INTRODUCTION: Pyridoxamine and pyridoxine are phosphorylated by pyridoxal kinase (EC 2.7.1.35) (1,2), then converted into pyridoxal 5′-phosphate (pyridoxal-P) by pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5) (3,4). Most pyridoxal-P is bound by proteins or hydrolyzed by phosphatases (1,3,5,6) and oxidized to pyridoxic acid (7,8). Snell and Haskell (9) first proposed that control of pyridoxal-P metabolism could occur by product inhibition of pyridoxamine-P oxidase and/or removal of pyridoxal-P by hydrolysis and oxidation. Li et al. (10) found that pyridoxal-P is more rapidly hydrolyzed when free than protein bound, and, hence, the tissue content of pyridoxal-P may be controlled by protein binding. The present study was undertaken to determine if product inhibition of pyridoxamine-P oxidase could additionally regulate the rate of pyridoxal-P formation.

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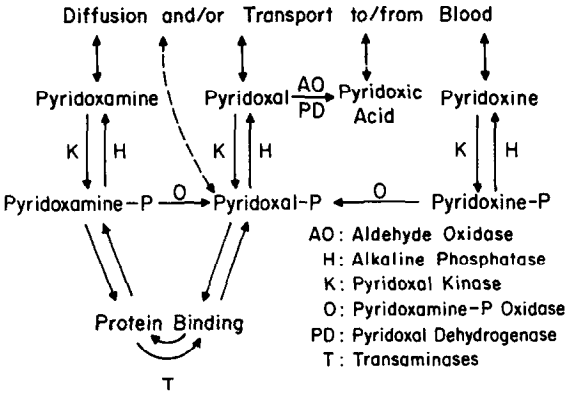
Major metabolic transformations of vitamin B_6 by liver.



MATERIALS AND METHODS: Liquid N_2-quick frozen livers from young adult, full-fed rabbits were obtained from Pel-Freez Biologicals (Rogers, AR). Rat livers were from male, Sprague-Dawley rats sacrificed immediately prior to use. The oxidase was purified according to Kazarinoff and McCormick (11). Pyridoxine-P was prepared by reduction of pyridoxal-P with sodium borohydride and purified by recrystallization. All other biochemicals were obtained from Sigma.

Livers were homogenized in the designated buffer containing 10 μM phenylmethylsulfonylfluoride by ten strokes of a hand-held TenBroeck apparatus. Cytosol was prepared by centrifuging the homogenate for one hr at 105,000g. All operations were conducted in dim light to prevent photodegradation of the flavin and pyridoxyl compounds.

Oxidase activities were determined by incubating the enzyme with substrate (5 ml final volume) at 37° in a gyrotory shaker (New Brunswick Scientific). After five to ten min intervals, aliquots were removed, added to trichloroacetic acid (5% final concentration), and pyridoxal and pyridoxal-P were separately quantitated according to Wada and Snell (4). Except where noted in the text, only pyridoxal-P was formed at early incubation times. Rates are initial velocities from plots of product formed versus time. One unit of oxidase activity represents the formation of one nmol of pyridoxal-P/assay/hr. The type of inhibition was ascertained by Lineweaver-Burk plots, but the values for the Michaelis-Menten parameters were determined by replotting the data according to Cornish-Bowden and Eisenthal (12).

RESULTS AND DISCUSSION: The Lineweaver-Burk plots for pyridoxal-P inhibition of pyridoxamine-P oxidase in a 10% homogenate of rabbit liver are shown in Figure 1. As for the pure enzyme, the inhibition is clearly competitive. These results, plus those for the pure enzyme and cytosol, are summarized in Table 1. Although the values for K_m vary somewhat, as has been seen previously (13) and probably reflects differences in the properties of the solvent (14), the values for K_i are insensitive to the presence of other cellular components. Only high

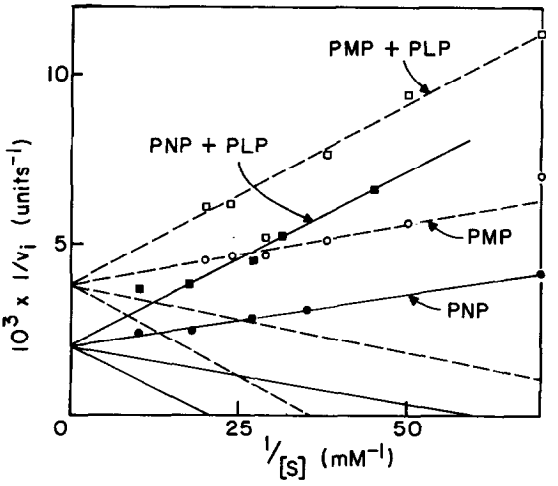


Figure 1: Lineweaver-Burk plots for oxidation of pyridoxamine-P (PMP) and pyridoxine-P (PNP) by a 10% homogenate of rabbit liver in the absence (o,•) and presence (□,■) of 5 μM pyridoxal-P (PLP). The lines are reflected onto the positive abscissa to show the intercepts.

TABLE 1. Michaelis Constants for Rabbit Liver Pyridoxamine-P Oxidase Under Various Conditions

Conditions	Substrate: Pyridoxamine-P K_m	Pyridoxamine-P K_i^{PLP*}	Pyridoxine-P K_m	Pyridoxine-P K_i^{PLP*}
	(μM)			
Pure enzyme in 0.2 M K phosphate (pH 8)	18	4	25	3
Pure enzyme in 0.2 M K phosphate (pH 7)	24	3	16	3
Pure enzyme in 0.5 M Tris-HCl (pH 8)	11	19	24	-
Cytosol (6 mg) in 0.2 M K phosphate (pH 8)	15	3	17	4
Homogenate (237 mg) in 0.2 M K phosphate (pH 8)	11	3	17	3

*Calculated for competitive inhibition.

concentrations of Tris, an amino group-containing buffer that forms a Schiff base with pyridoxal-P (4), increases the K_i. Neither H_2O_2 nor NH_3 inhibit activity at comparable concentrations.

The formation of pyridoxal-P from pyridoxine-P by a 10% homogenate of

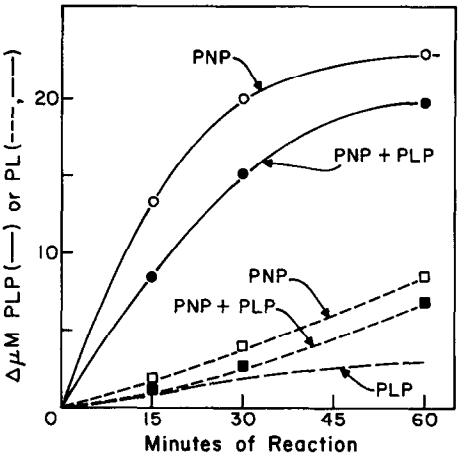


Figure 2: Time-course of formation of PLP and pyridoxal (PL) from 100 μM PNP by a 10% homogenate of rabbit liver in 50 mM HEPES (pH 7) in the absence (o,□) and presence (●,■) of 10 μM PLP. For ease of comparison, the content of PLP at zero-time was subtracted from subsequent determinations (ΔμM). The formation of PL from 10 μM PLP (——) is also included.

TABLE 2. Michaelis Constants for Rat Liver Pyridoxamine-P Oxidase Under Various Conditions*

Conditions	Substrate: Pyridoxamine-P K_m	Pyridoxamine-P V_{max}	Pyridoxine-P K_m	Pyridoxine-P V_{max}
	(μM)	(Units)	(μM)	(Units)
Cytosol (55 mg) in 0.2 M K phosphate (pH 8)	8	125	3	195
Cytosol plus 5 μM pyridoxal-P	20	150	10	245
Cytosol (55 mg) in 0.2 M K phosphate (pH 7)	--	--	8	260
Cytosol plus 5 μM pyridoxal-P	--	--	18	345
Homogenate (133 mg) in 0.2 M K phosphate (pH 8)	8	100	4	165
Homogenate plus 5 μM pyridoxal-P	39	140	22	220

*Since some hydrolysis of pyridoxal-P occurred, pyridoxal plus pyridoxal-P was used to calculate the velocity.

rabbit liver was examined using HEPES instead of phosphate buffer, since the latter inhibits alkaline phosphatase (6). As can be seen in Figure 2, 10 μM pyridoxal-P inhibited the initial velocity by 40%, as expected if removal of product was slow. Addition of 2 mM ATP and 0.5 mM $MgCl_2$ increased the inhibition slightly and reduced the apparent rate of hydrolysis of 10 μM pyridoxal-P by 20%. The rate of disappearance of 10 μM pyridoxal, which did not appear as pyridoxal-P and, hence, may reflect oxidation to pyridoxic acid, was 5.6 μM/hr.

Pyridoxamine-P oxidase from rat liver is also inhibited by product, as is shown in Table 2. Pyridoxal-P increased both K_m and V_{max}, the latter indicating distortion of the curve either by the more-active phosphatases of rat liver or due to difficulties in determining a true initial velocity when considerable product inhibition occurs at even early times. This prevents an exact estimation of K_i; however, the data clearly show that pyridoxal-P is a powerful inhibitor of the rat liver enzyme.

Inhibition of rabbit liver pyridoxamine-P oxidase by pyridoxal-P and other pyridoxyl compounds with sp^2 hybridization at carbon-4′ has been observed previously (4,11,15). However, as has also been pointed out (10,16), this inhibition would not be physiologically relevant if cellular components "trapped" pyridoxal-P by specific (e.g. by transaminases) and nonspecific binding or metabolism. The results reported here establish that such binding by a whole-liver homogenate does not take place and that the oxidase is strongly inhibited by pyridoxal-P. Similar inhibition was shown by the data of Li et al. (10), which involved the synthesis of pyridoxal-P by rat liver cytosol plus pyridoxine, ATP, $MgCl_2$, and phosphate (pH 7). Although they conclude that the oxidase was not involved because adding 50 μM pyridoxal-P did not alter the equilibrium level of pyridoxal-P formed from pyridoxine, their data actually showed that the rate of its formation was decreased by 40%, as expected for product inhibition of an O_2-coupled oxidase.

Whether or not inhibition of pyridoxamine-P oxidase regulates the in vivo rate of pyridoxal-P formation from pyridoxamine-P and pyridoxine-P will depend

on the relative and absolute concentrations of substrates and products. Both studies in vivo and in vitro (2,17-20) have found that pyridoxal kinase is relatively insensitive to product inhibition and maintains cellular pyridoxyl compounds primarily in the phosphorylated forms. Nonetheless, substrate concentrations are relatively low; even after administration of high levels (165 μg) of pyridoxine to mice, approximately 3.3 μg (16 nmol) of pyridoxine-P appears in the liver. Pyridoxal-P concentrations, on the other hand, may normally be significant. In addition to short-term accumulation of labeled pyridoxal-P after in vivo administration of radioactive pyridoxine (18,19), fairly high basal levels, 20 to 70 nmol/g wet weight (10,17,20,21), of pyridoxal-P are found in rat liver. Of this basal content, ~20% is readily removed by DEAE-cellulose chromatography (22), ~30% is removed by exhaustive dialysis (10), and 55 to 60% is removed (20) by apotryptophanase (K_m = 0.7 mM) (23). Although similar information is not available for rabbit liver, the B_6 content of rabbit liver (24) is sufficiently similar to the rat (25) that the pyridoxal-P content and distribution may be analogous in these species. Hence, from 4 to 14 nmol of pyridoxal-P is present/g wet weight in a free or loosely bound form.

Overall, these results establish that product inhibition of pyridoxamine-P oxidase is not merely a property of the purified enzyme but also can occur in vivo. Complete evaluation of the relative contributions of each step in B_6 metabolism awaits further work. However, the evidence thus far suggests that, whereas protein binding will determine the basal level of pyridoxal-P (10), pyridoxamine-P oxidase will play a kinetic role in regulating pyridoxal-P formation.

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