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INTERACTION OF TYROSINE PHENOL-LYASE WITH PYRIDOXAL PHOSPHATE *N*-OXIDE AND 2′-HYDROXY PYRIDOXAL PHOSPHATE

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SUMMARY

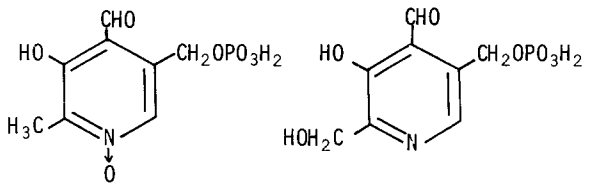
Pyridoxal phosphate *N*-oxide and 2′-hydroxy pyridoxal phosphate served as the coenzyme for tyrosine phenol-lyase (L-tyrosine phenol-lyase (deaminating), EC 4.1.99.2, formerly known as β-tyrosinase) from *Escherichia intermedia*. Reconstituted enzymes with these pyridoxal phosphate analogues exhibited an absorption band near 430 nm. In the presence of L-alanine, the enzyme associated with pyridoxal phosphate *N*-oxide has shown a very low absorption band at 500 nm compared with the native enzyme.

Tyrosine phenol-lyase (L-tyrosine phenol-lyase (deaminating), EC 4.1.99.2, formerly known as β-tyrosinase) of *Escherichia intermedia* has been crystallized in our laboratory[1] from cells of the organism and its properties established in some detail[2]. The enzyme was found to catalyze a series of α,β-elimination, β-replacement[3], racemization[4] and the reverse reaction of the α,β-elimination[5]. The crystalline preparation of tyrosine phenol-lyase was inactive in the absence of added pyridoxal-*P*. Binding of pyridoxal-*P* to the apoenzyme was accompanied by a pronounced increase in absorbance at 340 and 430 nm. The coenzyme forms an azomethine linkage with the ε-amino group of the lysine residue in the apoenzyme. The amount of pyridoxal-*P* bound to the apoenzyme has been determined to be 2 moles per mole (mol. wt 170 000) of enzyme[2].

The present paper reports the interaction of apotyrosine phenol-lyase with two pyridoxal-*P* analogues, pyridoxal-*P* *N*-oxide (Scheme Ia) and 2′-hydroxy pyridoxal-*P* (Scheme Ib), and the effect of modification on the catalytic activities of the resulting holoenzyme.

The incorporation of an oxygen atom to the pyridine nitrogen of pyridoxal-*P* has been described to decrease its electron-attracting ability and to lower the catalytic activities in several enzyme systems and model reactions[6–9]. The 2-methyl group of pyridoxal-*P* has been known not to play any catalytic role in non-enzymatic model reactions or in the reaction catalyzed by pyridoxal-*P* proteins but it plays an important spatial role in the hydrophobic interaction with the coenzyme

Scheme I



binding site of the apoenzyme[10]. The replacement of the 2-methyl group with a hydroxymethyl group resulted in a significant change in the interaction of the pyridoxal-*P* analogue with pyridoxal-*P* enzymes[11,12].

Pyridoxal-*P* *N*-oxide[6] and 2′-hydroxy pyridoxal-*P*[11] were synthesized according to the method previously reported. Crystalline tyrosine phenol-lyase was prepared from cells of *E. intermedia* grown in a bouillon-peptone medium containing L-tyrosine, according to the methods of Kumagai *et al.*[1]. Lactate dehydrogenase of rabbit muscle was purchased from Boehringer Mannheim, and NADH was purchased from Kyowa Hakko-kogyo Co. Ltd, Tokyo.

The enzyme activity was determined by measuring the formation of pyruvate from L-tyrosine at 30 °C by following the decrease in absorbance at 340 nm in the presence of excess NADH and lactate dehydrogenase. The reaction mixtures contained 5 μmoles of L-tyrosine, various amounts of pyridoxal-*P* or its derivatives, 0.066 μmole of NADH, lactate dehydrogenase (2.6 I.U. at 25 °C), 0.2 μmole of 2-mercaptoethanol and 200 μmoles of potassium phosphate buffer, pH 8.0, in a total volume of 2 ml. The amount of L-tyrosine was changed when it was necessary. The reaction was initiated by adding, from a micropipette, 0.02 ml of a solution containing 0.02–0.26 units[1] of tyrosine phenol-lyase, and mixing immediately. The decrease in absorbance at 340 nm, was read from the expanded scale of a spectrophotometer, Hitachi Double-beam spectrophotometer Model-124.

The reconstitution of tyrosine phenol-lyase was performed by dialysis of the apoenzyme against 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1 mM pyridoxal-*P* or its derivatives and 1 mM 2-mercaptoethanol, for 12 h at 5 °C. The absorption spectra of the holoenzyme species were measured by an automatic recording spectrophotometer, Shimazu MPS-50L, using the dialysis buffer as a reference.

Tyrosine phenol-lyase reconstituted with pyridoxal-*P* *N*-oxide and 2′-hydroxy pyridoxal-*P* have catalyzed the α,β-elimination reaction of L-tyrosine. Table I shows comparative coenzyme activities of pyridoxal and its analogues for tyrosine phenol-lyase. The $K_{coenzyme}$ (K_{co}) values for pyridoxal-*P* *N*-oxide and 2′-hydroxy pyridoxal-*P*, *i.e.* the concentrations of the analogues required for the half-maximum activity of the reconstituted enzyme were determined in each instance from double-reciprocal plots of the initial rate of pyruvate formation *vs* coenzyme concentration at 30 °C. The affinity of pyridoxal-*P* *N*-oxide for apotyrosine phenol-lyase was lower than that of pyridoxal-*P*. The extrapolated maximum velocity (v) of pyridoxal-*P* *N*-oxide activated enzyme was 80% of that of the native enzyme. The low coenzyme activity of pyridoxal-*P* *N*-oxide had been observed in tryptophanase[6], extramitochondrial

TABLE I

COMPARATIVE ACTIVITIES OF PYRIDOXAL-*P*, PYRIDOXAL-*P* *N*-OXIDE AND 2'-HYDROXY PYRIDOXAL-*P* AS COENZYMES FOR TYROSINE PHENOL-LYASE

K_{co} is defined as the concentration of pyridoxal-*P*, pyridoxal-*P* *N*-oxide, 2'-hydroxy pyridoxal-*P* required for a half-maximum activity of the reconstituted tyrosine phenol-lyase. The reaction conditions are written in the text.

Holoenzyme reconstituted with	*v* *(μmoles/min per mg)*	K_{co} $(\times 10^6\ M)$
Pyridoxal-*P*	2.2	2.2
Pyridoxal-*P* *N*-oxide	1.8	6.3
2'-Hydroxy pyridoxal-*P*	1.3	13

glutamate-oxaloacetate transaminase[6] and glutamate decarboxylase[13], and could be explained by the low electron-attracting ability of its pyridine nitrogen.

The K_{co} value of 2'-hydroxy pyridoxal-*P* for the apoenzyme was markedly larger than that of pyridoxal-*P*. The *v* value catalyzed by the 2'-hydroxy pyridoxal-*P*-bound enzyme was about 60% of that catalyzed by the native enzyme. Replacement of 2-methyl group of pyridoxal-*P* with a hydroxymethyl group has decreased the coenzyme activity of pyridoxal-*P* for the apotyrosine phenol-lyase. This behavior of the analogue is similar to that observed with *E. coli* tryptophanase[7]. For the extra-mitochondrial glutamat eoxaloacetate transaminase, 2'-hydroxy pyridoxal-*P* was found to have nearly equal K_{co} value to pyridoxal-*P* and a larger *v* than that of pyridoxal-*P*[7].

Holotyrosine phenol-lyase shows two absorption bands with maxima at 340 and 430 nm (Fig. 1-I). When pyridoxal-*P* is replaced by pyridoxal-*P* *N*-oxide, a major peak at 430 nm and a shoulder at 340 appeared (Fig. 1-II). A similar spectrum had been observed with pyridoxal-*P* *N*-oxide reconstituted tryptophanase[6].

The 2'-hydroxy pyridoxal-*P*-bound tyrosine phenol-lyase showed two similar bands with the pyridoxal-*P* enzyme (λ_{max} at 340 and 425 nm, Fig. 1-III), but the

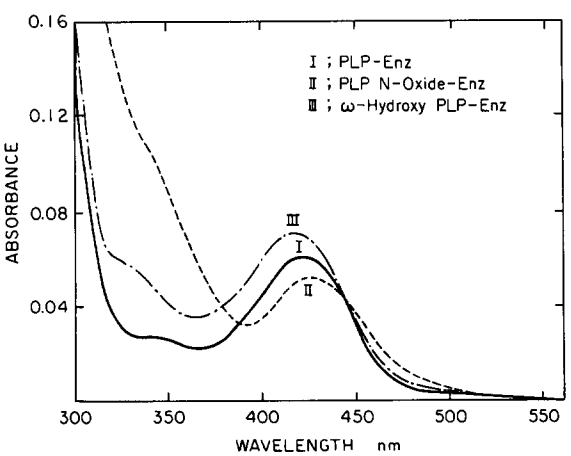


Fig. 1. Absorption spectra of tyrosine phenol-lyase reconstituted with pyridoxal-*P*(PLP)(I), pyridoxal-*P*(PLP) *N*-oxide(II) and 2'-hydroxy pyridoxal-*P*(PLP)(III). The enzyme (1.11 mg of protein) was dissolved in 1.0 ml of 0.1 M potassium phosphate buffer, pH 8.0. The conditions of reconstitution and the measurement are written in the text.

intensities of both peaks were higher than those observed with the native enzyme.

The absorption peak near 430 nm of the reconstituted enzymes suggests that both analogues, pyridoxal-*P* *N*-oxide and 2′-hydroxy pyridoxal-*P*, bind with the apotyrosine phenol-lyase through an azomethine linkage in the same manner as that with the native coenzyme, pyridoxal-*P*.

On addition of the substrates or competitive inhibitors such as L-alanine, tyrosine phenol-lyase exhibits an absorption maximum near 500 nm[2] (Fig. 2-I). Morino and Snell[14] had reported the similar spectral behavior of tryptophanase of *E. coli* and ascribed the 500-nm band to a quinonoid intermediate that lacks the α-proton of the bound amino acid. The absorption at 500 nm was observed by the addition of L-alanine to the pyridoxal-*P* *N*-oxide-bound enzyme, but the intensity was very low in comparison with the pyridoxal-*P* enzyme (Fig. 2-II). A possible

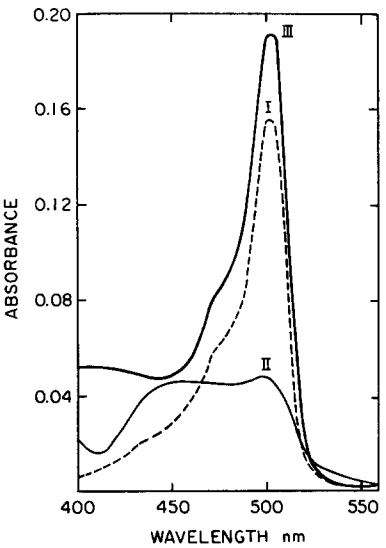


Fig. 2. Absorption spectra of tyrosine phenol-lyase reconstituted with pyridoxal-*P*(PLP)(I), pyridoxal-*P*(PLP) *N*-oxide(II) and 2′-hydroxy pyridoxal-*P*(PLP)(III) in the presence of L-alanine. The spectra were taken with solutions containing 0.62 mg of enzyme (Spectrum I) or 1.1 mg of enzyme (Spectrum II and III) in 1.0 ml of 0.1 M potassium phosphate buffer, pH 8.0. L-Alanine (33.3 μmoles) was added to the enzyme solution and the dialysis buffer was used as a reference. The conditions of the reconstitution and the measurement are written in the text.

explanation of this observation is that the decrease in the electronegativity of the analogue stabilizes the ES complex of the aldimine type and does not favor the electron shift necessary for the subsequent deprotonation reaction[6].

Tyrosine phenol-lyase reconstituted with 2′-hydroxy pyridoxal-*P* has shown quite a similar peak at 500 nm with the pyridoxal-*P*-bound enzyme by the addition of L-alanine (Fig. 2-III).

REFERENCES

1 Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. and Ogata, K. (1970) *J. Biol. Chem.* 245, 1767–1772

2 Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. and Ogata, K. (1970) *J. Biol. Chem.* 245, 1773–1777
3 Kumagai, H., Matsui, H., Ohgishi, H., Ogata, K., Yamada, H., Ueno, T. and Fukami, H. (1969) *Biochem. Biophys. Res. Commun.* 34, 266–270
4 Kumagai, H., Kashima, N. and Yamada, H. (1970) *Biochem. Biophys. Res. Commun.* 39, 796–801
5 Yamada, H., Kumagai, H., Kashima, N., Torii, H., Enei, H. and Okumura, S. (1972) *Biochem. Biophys. Res. Commun.* 46, 370–374
6 Fukui, S., Ohishi, N., Nakai, Y. and Shimizu, S. (1969) *Arch. Biochem. Biophys.* 130, 584–593
7 Masugi, F., Natori, Y., Shimizu, S. and Fukui, S. (1973) *J. Nutr. Sci. Vitaminol.* 19, 55–70
8 Fukui, S., Ohishi, N. and Shimizu, S. (1970) in *Methods in Enzymology* (McCormick, D. B. and Wright, L. D., eds), Vol. 18A, pp. 598–603, Academic Press, New York and London
9 Ivanov, V. I. and Karpeisky, M. Ya. (1969) in *Advances in Enzymology* (Nord, F. F., ed.), Vol. 32, pp. 21–53, Interscience Publishers, New York, London, Sydney and Toronto
10 Morino, Y. and Snell, E. E. (1967) *Proc. Natl. Acad. Sci. U.S.* 57, 1692–1699
11 Fukui, S., Ohishi, N., Nakai, Y. and Masugi, F. (1969) *Arch. Biochem. Biophys.* 132, 1–7
12 Fukui, S., Nakai, Y. and Masugi, F. (1970) in *Methods in Enzymology* (McCormick, D. B. and Wright, L. D., eds), Vol. 18A, pp. 603–606, Academic Press, New York and London
13 Fonda, M. L. (1971) *J. Biol. Chem.* 246, 2230–2240
14 Morino, Y. and Snell, E. E. (1967) *J. Biol. Chem.* 242, 2800–2809