INTERACTION OF THE APOENZYME OF L-GLUTAMATE DECARBOXYLASE WITH PYRIDOXAL PHOSPHATE ANALOGUES

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1. Introduction

Enzymes requiring pyridoxal-5′-phosphate as cofactor catalyze a variety of reactions of amino acid metabolism, including transamination and decarboxylation. To appreciate the role of different parts of the pyridoxal-5′-phosphate molecule both in its binding to the protein and in the enzymic reaction, the interactions of apo-L-glutamate decarboxylase with analogues of pyridoxal-5′-phosphate modified at positions 2, 3, 4, 5 and 6 of the pyridine ring have been studied*.

2. Materials and methods

Pyridoxal-5′-phosphate analogues were synthesized according to previously published methods [2, 3]. Glutamate decarboxylase was isolated from cells of *E. coli* 600 M and purified by the procedure of Shukuya and Schwert [4]. Enzymic activity was routinely assayed by the conventional Warburg technique at 37° and at pH 4.6 [4]. The apoenzyme, prepared by the modified procedure of Huntley and Metzler [5], retained 1–4% of original activity and, on addition of an excess of pyridoxal-5′-phosphate at pH 4.6 was 90–100% reactivated. In most experiments, preliminary reduction with $NaBH_4$ was used to quench residual activity of the apoenzyme.

To study reactivation by pyridoxal-5′-phosphate analogues, the apoenzyme was preincubated with an excess of the analogue for 30–60 min at 0° in 0.1 M pyridine-HCl buffer, pH 4.6. The inhibition of a combination of the apoenzyme with the natural coenzyme by catalytically inactive pyridoxal-5′-phosphate analogues was determined as follows: 3 ml of pyridine-HCl buffer, pH 4.6, containing pyridoxal-5′-phosphate (5×10^{-6} M), pyridoxal-5′-phosphate analogue (3.3×10^{-4} M) and apoenzyme ($\sim 1 \times 10^{-7}$ M), was placed in the main chamber of Warburg vessel; after incubation of this mixture for 5 min at 37°, 0.2 ml of 1×10^{-2} M L-glutamate was tipped in from the side arm and the initial rate of decarboxylation was measured. K_i values were estimated under similar conditions by the method of Dixon**. For determination of the dissociation constant, K_{Co}, of pyridoxal-5′-phosphate or its analogues, giving noticeable reactivation effects, a dilute solution of apoenzyme ($\sim 1 \times 10^{-7}$ M) was incubated at 4° with 3–6 equivalents of coenzyme (or analogue) until equilibrium had been reached. Concentrations of apo- and holoenzyme in equilibrium mixture were determined on the basis of enzymic activity and the K_{Co} value was calculated as follows:

$$K_{Co} = [\text{apoenzyme}] \times [\text{cofactor}]/[\text{holoenzyme}]$$

* Part of this work has been reported at the 8th International Congress of Biochemistry [1].

** The K_i values thus obtained possibly do not represent true dissociation constants (because Dixon's method was elaborated to study the inhibition of enzymes by substrate analogues), but they are presumably proportional to dissociation constants.

Table 1
Reactivating and inhibitory activities of pyridoxal-*P* analogues.

Analogues of PLP*	Degree of reactivation** (% of PLP reactivation)	Degree of inhibition, (%)	K_i (M)
PNP	–	76	2×10^{-5}
4-deoxy PNP	–	46	–
PMP	–	10	$\sim 2 \times 10^{-3}$
3-O-Me-PLP	4	63	–
3-deoxy-PLP	0.6	35	1×10^{-4}
2-nor-PLP	20	–	–
2′, 2′-diMe-PLP	0	21	–
2′-*n*-Propyl-PLP	0	35	2×10^{-4}
6-Me-PLP	4	65	7×10^{-5}
2-nor-6-Me-PLP	5.5	69	–
5′-Me-PLP	20	–	–
5-nor-5-β-Carboxyethylpyridoxal	0	30	3×10^{-4}
5-nor-5-β-Carboxyvinylpyridoxal	0	60	–
5′-Deoxypyridoxal	0	19	4×10^{-4}

* *Abbreviations*: PLP pyridoxal-5′-phosphate; PMP pyridoxamine-5′-phosphate; PNP pyridoxine-5′-phosphate.
** The maximum degree of reactivation is presented, as obtained on addition of 3000 equivalents of 3-*O*-methyl PLP, 1500 equivalents of 3-deoxy-PLP, 6-Me-PLP or 2-nor-6-Me-PLP and 100–200 equivalents of 2-nor-PLP or 5′-Me-PLP per one active site of the protein.

Table 2
Properties of the complexes of apodecarboxylase with pyridoxal-5′-phosphate and its analogues.

Compounds	K_{Co} (M)	K_m for L-glutamate	V_{max} (μM CO_2/min/mg prot.)	Circular dichroism at 420 nm ϵM
PLP	1×10^{-7}	9×10^{-4}	180	+ 6.6
2-nor-PLP	1.4×10^{-6}	5.5×10^{-4}	34	+ 5.1
5′-Methyl-PLP*	2.5×10^{-6}	1.5×10^{-3}	45	+ 6.6

* Racemic preparation.

3. Results and discussion

The results of this study are presented in table 1. The data show that, of coenzyme analogues tested, pyridoxine-5′-phosphate and pyridoxamine-5′-phosphate possess, respectively, the highest and the lowest affinity for the active site of L-glutamate decarboxylase. This remarkable difference is reasonably ascribed to an interaction of the positive charge of the 4′-ammonium group of pyridoxamine-5′-phosphate with the protein. This positive charge may be repelled by the ε-ammonium group of the active site lysyl residue [6, 7].

The experiments with 3-*O*-Me-pyridoxal-5′-phos-

phate and 3-deoxypyridoxal-5′-phosphate have shown that the phenolic hydroxyl group is essential both for binding of the coenzyme to the protein and for catalysis of decarboxylation. 3-Deoxypyridoxal-5′-phosphate has no reactivation effect even at concentrations at which it has an inhibitory effect, i.e. binds to the protein. A very slight reactivation observed in the presence of a 3,000-fold excess of 3-*O*-methylpyridoxal-5′-phosphate is possibly due to a trace amount of pyridoxal-5′-phosphate in the analogue sample. It is noteworthy that the K_i value for 3-deoxypyridoxal-5′-phosphate is much larger than for pyridoxine-5′-phosphate and thousandfold larger than the K_{Co} for pyridoxal-5′-phosphate. From these results, it seems probable that 3-deoxypyridoxal-5′-phosphate does not form an aldimine bond with the ϵ-amino group of the protein, since it is unlikely that the lack of 3-hydroxy group per se could explain the drastic decrease in affinity of the cofactor analogue for the apoenzyme.

Among the coenzyme analogues modified at position 2 of the pyridine ring, only 2-norpyridoxal-5′-phosphate exhibits noticeable reactivating activity with apoenzyme. 2-Propylpyridoxal-5′-phosphate and 2′,2′-diMe-pyridoxal-5′-phosphate slightly inhibit specific binding of pyridoxal-5′-phosphate to the protein, but do not reactivate the apodecarboxylase even at concentrations at which they bind to the protein. The data of table 2 show that removal of the 2-methyl group leads to a marked augmentation of the K_{Co} value, but has almost no effect on the K_m value of the artificial cofactor–protein complex for L-glutamate, although the V_{max} value is decreased fivefold. These data indicate that the 2-methyl group of pyridoxal-5′-phosphate is important not only for binding of the cofactor to the protein, but likewise for catalytic activity of the holoenzyme. The complex of apodecarboxylase with 2-norpyridoxal-5′-phosphate exhibits, at pH 4.6, a positive circular dichroism band at 420 nm, slightly lower than that of normal holoenzyme.

Introduction of a methyl group at position 6 of the pyridine ring markedly decreases the affinity of the coenzyme for apodecarboxylase. Complexes prepared by reconstitution of the apoenzyme with 6-methylpyridoxal-5′-phosphate and 2-nor-6-methylpyridoxal-5′-phosphate display 4 and 5.5%, respectively, of enzymic activity of the normal holoenzyme. It well may be that the 6-methyl group interferes with correct spatial orientation of the coenzyme in the active site. Upon $NaBH_4$-treatment of the complex of apodecarboxylase with 6-methylpyridoxal-5′-phosphate, addition of pyridoxal-5′-phosphate has no reactivation effect. This strongly suggests that 6-methyl-pyridoxal-5′-phosphate forms an aldimine bond with the apoenzyme.

Among the coenzyme analogues modified at position 5 of the pyridine ring, only 5′-methylpyridoxal-5′-phosphate displays coenzyme activity. The V_{max} value of the complex of apodecarboxylase with this analogue is fourfold smaller than that of the normal holoenzyme. Both complexes have identical positive circular dichroism bands at 420 nm; their K_m values for L-glutamate differ insignificantly.

The results of our experiments may be summarized as follows: even small changes in the coenzyme structure drastically decrease affinity of the analogue for apodecarboxylase and markedly reduce or fully eliminate the catalytic efficiency of the enzyme. It is of interest to compare the reactivation effects of pyridoxal-5′-phosphate analogues for apo-L-glutamate decarboxylase with those for the apoenzymes of arginine decarboxylase [9] and of aspartate aminotransferase [8]. In the case of the aminotransferase all alkyl analogues of pyridoxal-5′-phosphate are bound to the protein giving artificial enzymes with fairly high activities. The V_{max} value of 2-norpyridoxal-5′-phosphate aminotransferase is even higher than that of natural holoenzyme [8]. The same is true for arginine decarboxylase [9]. In the latter case, the V_{max} value of 2-norpyridoxal-5′-phosphate complex is higher than that of natural enzyme, while V_{max} of the 6-methylpyridoxal-5′-phosphate complex is half that of the normal enzyme. It thus appears that, of the pyridoxal-5′-phosphate dependent enzymes so far tested, glutamate decarboxylase makes the most stringent demands as to structure of the coenzyme. It is known that both the 2-methyl and 5′-phosphate groups of pyridoxal-5′-phosphate are dispensable for non-enzymic catatalysis of decarboxylation of amino acids. On the other hand, our data show that these substitutents influence not only the affinity for the apoenzyme, but also, and to a remarkable extent, the catalytic efficiency of the artificial holoenzymes. This fact can be explained by the suggestion that the substitutents mentioned above contribute to appropriate adjustment of the active site and/or to necessary reorientations of coenzyme–substrate intermediates

in the course of enzymic decarboxylation.

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