INTERACTION OF L-α-METHYL-α-HYDRAZINO-3,4 DIHYDROXYPHENYLPROPIONIC ACID WITH DOPA-DECARBOXYLASE FROM PIG KIDNEY

Carla BORRI-VOLTATTORNI and Alba MINELLI
Institute of Biological Chemistry, Faculty of Pharmacy

and

Piero BORRI
Institute of Nervous and Mental Diseases, Faculty of Medicine, University of Perugia, 06100 Perugia, Italy

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1. Introduction

Considerable evidence has now been accumulated indicating that the symptoms of Parkinsonism are related to a depletion of striatal dopamine. Since dopamine does not cross the blood–brain barrier, efforts to correct this deficit have focused on the administration of L-Dopa, its immediate precursor. Some of the principal complications of L-Dopa administration are related to the peripheral formation of catecholamines by the decarboxylation of L-Dopa. One refinement in its use involves administration, in addition to Dopa, of compounds which selectively inhibit the metabolism of the precursor in peripheral tissues but not in brain. These products, which are peripheral Dopa-decarboxylase inhibitors and do not appear to pass the blood–brain barrier, should reduce the peripheral side effects of L-Dopa without interfering with its central action. Recently, since the purification of Dopa-decarboxylase from pig kidney by various authors [1–3], considerable progress has been made towards the knowledge of its specificity, inhibition, spectral properties and the mode of binding of the coenzyme and the substrates [1,4,5].

This paper deals with the interaction of a homogeneous preparation of Dopa-decarboxylase from pig kidney with a compound of current interest in Parkinson's disease: the L-α-methyl-α-hydrazino-3,4-dihydroxyphenilpropionic acid (HMD). The results show an high affinity of the enzyme for this product and provide a spectral evidence of enzyme–HMD complex formation.

2. Experimental

Dopa-decarboxylase (EC 4.1.1.26) has been purified from pig kidney according a method, previously reported [3], very close to that of Lancaster et al. [2]. The enzyme is shown to be homogeneous by the criteria of disc-gel electrophoresis and ultracentrifugation [6]: its specific activity is found equal to that previously reported [2] and its coenzyme content [4] is in excellent agreement with Christenson et al. [1]. All the compounds used were purchased from Sigma Chem. Co. HMD was supplied by Merck Sharp and Dohme.

The assay method was by Sherald et al. [7] according to the modification introduced by Charteris et al. [8]. Rates of decarboxylation were determined in a reaction mixture containing substrate and inhibitor in the required amounts and 0.05 M K-phosphate buffer, pH 6.8. The reaction was started by addition of the enzyme. The solutions were incubated for 5 min for L-Dopa and 10 min for 5-hydroxy-L-tryptophan (L-5HTP) at 25°C. One unit of enzyme activity is definied as the amount of protein which catalizes the production of 1 nmol amine/min.

Spectral data were obtained in a DB-GT Beckman spectrophotometer connected to a Beckman 10 inch recorder.

3. Results and discussion

HMD is such a powerful inhibitor of pig kidney Dopa-decarboxylase with respect to L-Dopa and L-5HTP that the inhibition is observed when the molarities of inhibitor and enzyme are of the same order, as shown in fig.1.

Inhibition of decarboxylase activity against 4.4×10^{-4} M L-Dopa and 4×10^{-4} M L-5HTP is also tested at several concentrations of enzyme at a fixed HMD concentration. The percent inhibition at 0.4×10^{-7} M HMD concentration ranges from 54–24% for L-Dopa and at 1.2×10^{-7} M HMD concentration it ranges from 82–40% for L-5HTP when the enzymic concentration varies from 0.35–1.77×10^{-7} M and 1.42–4.26×10^{-7} M, respectively. From these results it can be inferred that the degree of inhibition is dependent not only upon the amount of inhibitor but upon the amount of enzyme present: the percentage inhibition decreases with increasing enzyme concentration. These data give little information in terms of the mechanism of inhibition because, under the experimental conditions of the assay actually used to determine the decarboxylation rate, the inhibitor concentration is not greatly in excess in comparison with the enzyme concentration, as generally assumed in the classical kinetic treatments of enzyme inhibition.

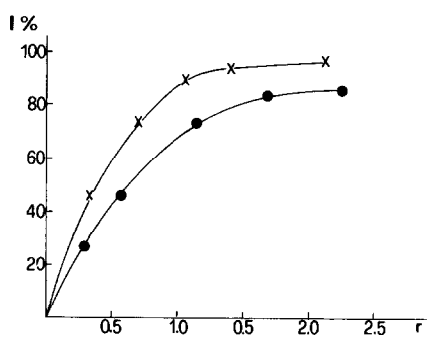

Fig.1. Inhibition of Dopa-decarboxylase activity as a function of inhibitor to enzyme ratio using L-Dopa (o———o), and L-5HTP (X———X) as substrates at 4.4×10^{-4} M and 4×10^{-4} M concentration, respectively. The concentration of enzyme in the assay mixtures was constant (1.42×10^{-7} M) in both cases. *r* is the molar ratio HMD/enzyme.

It is a inhibition characterized by an enzyme–inhibitor complex formation theoretically reversible but with an apparent dissociation constant so small that the percent inhibition varies with the amount of the enzyme. Thus, at concentrations of inhibitor inadequate to cause complete inhibition of the enzyme, pratically all the inhibitor is enzyme-bound.

Spectrophotometric studies have been carried out in order to investigate the interaction of HMD, even in absence of substrate, with Dopa-decarboxylase.

The spectral change observed upon addition of this inhibitor to an enzyme solution, at pH 6.8, is characterized by a new absorption band at 395 nm whose intensity increases with the inhibitor binding. The appearance of an isosbestic point at about 435 nm gives evidence of a complex formation (fig.2).

Exhaustive dialysis of 10.49×10^{-6} M enzyme and 9.85×10^{-6} M HMD against 0.1 M K-phosphate buffer, pH 6.8, containing 1×10^{-4} M dithiotreitol leaves this spectrum unmodified. Spectrophotometric titration of enzyme with HMD has been carried out by using the absorption change at 395 nm as the criterion for complex formation. Data reported in the inset of fig.2 show a sharp titration curve with an end-point value reached at a ratio of 0.84 mol HMD/mol enzyme. The same behaviour is observed when the titration is performed on the enzyme complexed with L-5HTP at saturating concentration.

Addition of substrate to an enzyme–inhibitor solution at a 0.84 molar ratio does not change the spectral properties. Spectral data show that the 420 nm absorbing form is converted into a peak absorbing at 395 nm, probably due to an aldazone formation. The sharp titration indicates a very tight binding of HMD and enzyme either in absence or in presence of substrate. The end-point value of the enzyme titration does confirm the pyridoxal-P-content, as calculated by Christenson [1] and Borri [4]. HMD is an excellent compound to estimate the molar Dopa-decarboxylase concentration of unknown enzymic solutions.

The rate of formation of HMD–enzyme complex at 10°C is so fast that we cannot follow it, while in presence of L-5HTP or L-Dopa enzyme complexes it is accomplished in almost 2 min.

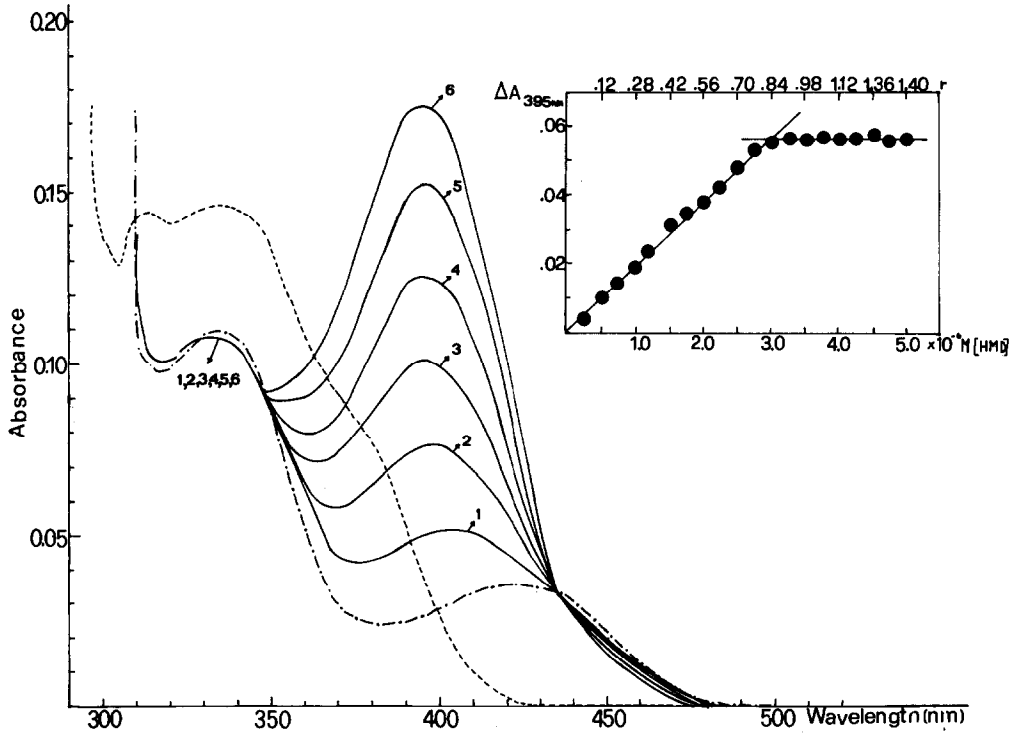

Fig.2. Spectra in K-phosphate buffer, pH 6.8 (– · –) enzyme (1.85×10^{-5} M). (———) Enzyme plus varying HMD concentrations: (1) 1.26×10^{-6} M, (2) 2.63×10^{-6} M, (3) 3.95×10^{-6} M, (4) 5×10^{-6} M, (5) 6.64×10^{-6} M, (6) 7.85×10^{-6} M. (– – –) Pyridoxal-P 1×10^{-5} M plus HMD 1×10^{-4} M. In the inset: binding curve of HMD to the enzyme (3.55×10^{-6} M) measured by the changes in absorbance at 395 nm. *r* is the molar ratio HMD/enzyme.

The inhibitor–enzyme complex we have observed is quite different from the hydrazone formed with free pyridoxal-P characterized by a shoulder around 380 nm and two absorption bands at 335 nm and 315 nm (fig.2). The hydrazone formation between 1×10^{-4} M HMD and 1×10^{-5} M pyridoxal-P has been monitored at 335 nm and a $t_{1/2}$-value of 1.19 min was calculated.

The high affinity of enzyme to HMD is not unexpected if its chemical structure is considered. HMD is a compound provided with the right requirements for a good interaction with Dopa-decarboxylase: this molecule has at least three suitable points for attachment to the enzyme: a catechol ring, a carboxylic group and a hydrazinic one which binds to the aldeidic function of the coenzyme. This aldeidic group which exists as a Shiff base in Dopa-decarboxylase is more reactive ($t_{1/2} \ll 5$ s) than the free aldeidic group of pyridoxal-P ($t_{1/2}$ = 1.19 min) to a nucleophilic attack, as already stated for glutamic–aspartic transaminase from pig heart [9]. The 420 nm absorbing form, attributed to a protonated Schiff base [4], reacts with HMD proving to be the most reactive species towards the carbonyl reagents, according to Cordes and Jencks [10].

The different inhibition percentage towards L-Dopa and L-5HTP (fig.1) is not easily explained: the reaction of binding of HMD to enzyme appears indeed to be fast as shown by the fact that a preincubation of enzyme with the inhibitor does not affect the activity towards substrates with very similar K_m-values but different V_{max} [1]. One possibility is that this behaviour could be related to the enzyme's multiplicity of sites where substrate and inhibitor molecules could bind without affecting the absorption peaks of the enzyme-bound pyridoxal-P, as already hypothesized [11,12]. The observed lowered rate of enzyme–inhibitor complex formation in presence of enzyme–

substrate complex suggests a displacement of the substrate by the inhibitor, while the inhibitor, once combined with all the enzyme, cannot be effectively displaced by substrate.

If the inhibitor affects enzyme activity to an extent that depends upon the enzyme concentration, when patients with Parkinson's disease are given this inhibitor, a tissutal selective inhibition of decarboxylase can come out. A tissue containing a small amount of Dopa-decarboxylase might have enzyme completely inhibited by an inhibitor amount which would inactivate only a small fraction of the enzyme in tissues containing larger amounts of the enzyme.

At present we do not know if therapeutic doses, administration times and presence of substrates in various tissues lead to a complete or selective peripheral-Dopa-decarboxylase inhibition.

Further effects on the binding of the inhibitor to the enzyme could be produced by other reacting components in tissues. All pyridoxal-P enzymes are known to be able to interact with hydrazinic groups and, as we have shown, pyridoxal-P reacts with HMD.

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