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2,5-DIHYDRO-L-PHENYLALANINE: A COMPETITIVE INHIBITOR OF INDOLEAMINE 2,3-DIOXYGENASE AND TRYPTOPHAN 2,3-DIOXYGENASE*

Yasuyoshi Watanabe, Motokazu Fujiwara§ and Osamu Hayaishi+

Department of Medical Chemistry,
Kyoto University Faculty of Medicine, Kyoto 606, Japan

and

Tomio Takeuchi and Hamao Umezawa

Institute of Microbial Chemistry,
3-14-23, Kamiosaki, Shinagawaku, Tokyo 141, Japan

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Summary

2,5-Dihydro-L-phenylalanine, an inhibitor of tryptophan 5-monooxygenase, was shown to be a potent inhibitor of indoleamine 2,3-dioxygenase (K_i = 0.23 mM) and tryptophan 2,3-dioxygenase (K_i = 0.70 mM), whereas tryptophan side chain oxidase from Pseudomonas was not inhibited by this compound.

A variety of enzyme inhibitors for metabolic processes in animals have been found in culture filtrates of microorganisms (1-3). In search for an inhibitor of brain metabolism, Okabayashi et al. isolated three strains of actinomycetes which produced the inhibitors of tryptophan 5-monooxygenase (E.C. 1.14.16.4) from

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§ Present address, Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606, Japan.

+ To whom all correspondence should be addressed.

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bovine pineal gland (4). One of them was isolated and found to be identical with 2,5-dihydro-L-phenylalanine (DiHPhe), which had been chemically synthesized previously (5). Kinetic analyses indicated that DiHPhe was a competitive inhibitor of tryptophan 5-monooxygenase with L-tryptophan (K_i = 44 μM) (4). In order to extend the finding to other enzymes that metabolize L-tryptophan, kinetic studies are presented with the following three heme-containing enzymes.

Indoleamine 2,3-dioxygenase catalyzes the oxygenative ring cleavage of not only D- and L-tryptophan but also many other substituted and unsubstituted indoleamines (6, 7). This enzyme was distributed in the intestine, lung, stomach, spleen, and brain of the rabbit (8). Recent studies from our laboratory have demonstrated that superoxide anion (O_2^-) is involved in the catalytic process of the enzyme both *in vitro* (9-12) and *in vivo* (13).

Tryptophan 2,3-dioxygenase (L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing), E.C. 1.13.11.11) catalyzes the incorporation of molecular oxygen to L-tryptophan to yield formyl-L-kynurenine. The enzyme has been isolated from animal liver (14) and *Pseudomonas* (15).

Tryptophan side chain oxidase, another heme-containing enzyme purified and crystallized from *Pseudomonas* in our laboratory (16), catalyzes the oxidation of the side chain of tryptophan and other indole derivatives.

The effect of DiHPhe on these three enzymes and the results of kinetic studies are reported in this paper.

Materials and Methods

2,5-Dihydro-L-phenylalanine hydrochloride was isolated from a culture filtrate of a streptomycete strain ME 238-AG 4 as pre-

viously reported (4). L-Tryptophan, L-ascorbic acid and methylene blue were obtained from Wako Pure Chemical Industries. Catalase was the product of Boehringer Mannheim. Hematin, L-phenylalanine and L-tyrosine were purchased from Sigma. L-[carboxy-^{14}C]Tryptophan (50 mCi/mmol) was obtained from New England Nuclear. All other chemicals were of reagent grade.

Indoleamine 2,3-dioxygenase was purified about 400-fold from crude extracts of rabbit small intestine by the method of Shimizu et al. (7) except that the isoelectric focusing step was omitted. The ratio of absorbance at 406 nm to that at 280 nm was 1.74 and the specific activity was 1.63 μmol/min/mg protein at 25° with L-tryptophan as substrate.

Tryptophan 2,3-dioxygenase was partially purified from the supernatant fraction (10^5 X g, 60 min) of liver homogenates of mice which were pre-treated by intraperitoneal injection of hydrocortisone and L-tryptophan. The purification (about 120-fold) was achieved by ammonium sulfate fractionation, Bio-Gel A-0.5 m and DEAE-cellulose column chromatographies, and the eluate was concentrated with ammonium sulfate**. Throughout the purification, L-tryptophan was present to prevent inactivation of the enzyme (17). Prior to kinetic studies, the enzyme preparation was passed through a Sephadex G-25 column to remove L-tryptophan. The specific activity of the purified enzyme was 0.09 μmol/min/mg protein at 25° with L-tryptophan as substrate.

Assay of these two enzymes was performed spectrophotometrically at 25° with a Shimadzu recording spectrophotometer model UV-300 in a cuvette with a 1-cm light path. The initial rate of reaction was determined for indoleamine 2,3-dioxygenase activity and the reaction rate from 10 to 20 min for tryptophan 2,3-dioxygenase activity because of the presence of lag phase (18).

The reaction mixture for indoleamine 2,3-dioxygenase (1.0 ml) contained 50 mM potassium phosphate, pH 6.6, 25 μM methylene blue, 10 mM ascorbic acid, 250 μg of catalase, various concentrations of L-tryptophan and DiHPhe, and 4.89 μg of enzyme. That for tryptophan 2,3-dioxygenase (1.0 ml) contained 50 mM potassium phosphate, pH 7.0, 0.5 μM hematin, 1.0 mM ascorbic acid, various concentrations of L-tryptophan and DiHPhe, and 168 μg of enzyme.

Tryptophan side chain oxidase from Pseudomonas was a generous gift of Drs. K. Takai and Y. Noda of this laboratory. The preparation had a specific activity of 1.4 μmol/min/mg protein at 25° with L-tryptophan as substrate. The enzyme activity was followed by measuring the rate of $^{14}CO_2$ evolution with L-[carboxy-^{14}C]tryptophan as substrate (16). The assay mixture (2.0 ml) contained 50 mM potassium phosphate, pH 6.0, 10 μM L-[carboxy-^{14}C]tryptophan (300 cpm/nmol), varying concentrations of DiHPhe and 0.3 μg of enzyme.

Protein was estimated according to Lowry et al. (19) using bovine serum albumin as standard.

Thin layer chromatography was carried out on cellulose precoated glass plates with a solvent system of 1-propanol:H_2O (7:3).

** Y. Watanabe, M. Fujiwara, and O. Hayaishi, unpublished data.

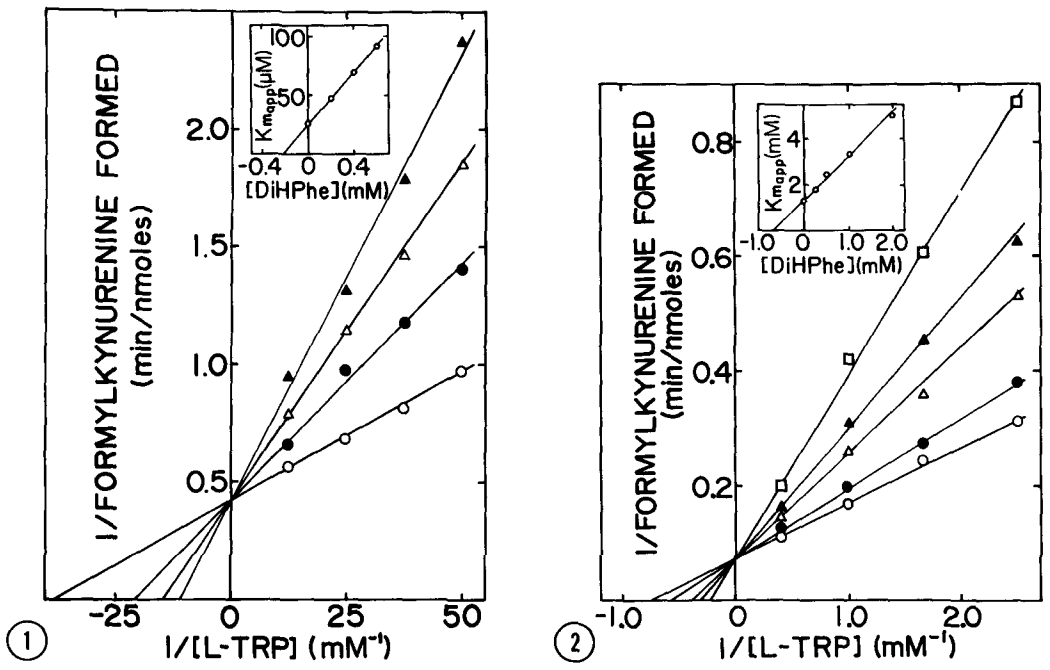

Fig. 1. Double reciplocal plots of the rates of the reaction against L-tryptophan concentrations at varied concentrations of DiHPhe with indoleamine 2,3-dioxygenase. DiHPhe, O mM, o——o; 0.2 mM, ●——●; 0.4 mM, Δ——Δ; 0.6 mM, ▲——▲. The assay was performed as described under "Materials and Methods". Inset; replots of the same results.

Fig. 2. Double reciplocal plots of the rates of the reaction against L-tryptophan concentrations at varied concentrations of DiHPhe with mouse liver tryptophan 2,3-dioxygenase. DiHPhe, O mM, o——o; 0.25 mM, ●——●; 0.5 mM, Δ——Δ; 1.0 mM, ▲——▲; 2.0 mM, □——□. The assay was performed as described under "Materials and Methods". Inset; replots of the same results.

Results and Discussion

2,5-Dihydro-L-phenylalanine inhibited both indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. Double reciplocal plots of the results shown in Figs. 1 and 2 indicate that the inhibition was linear competitive with L-tryptophan. The K_i values obtained from the replots of the same data were 0.23 mM and 0.70 mM, while the K_m values for L-tryptophan were 0.026 mM and 1.3 mM, respectively.

Although DiHPhe was shown to undergo nonenzymatic conversion to L-phenylalanine (5, 20), no trace of L-phenylalanine was formed upon storage of DiHPhe in H_2O for 30 min at 25° as judged by thin layer chromatography. When DiHPhe was incubated with either of these two enzymes for 20 min at 25°, spectral changes were scarcely observed. Neither L-phenylalanine (20 mM), tyrosine (2 mM), alanine (10 mM) nor histidine (10 mM) had any inhibitory effect on both enzymes.

Tryptophan side chain oxidase was not inhibited by 10 mM DiHPhe in the presence of 10 μM L-tryptophan. Okabayashi et al. reported that DiHPhe did not inhibit aromatic amino acid decarboxylase using 5-hydroxy-L-tryptophan as substrate (4).

These data suggested that DiHPhe inhibits the enzymes that metabolize the indole moiety of tryptophan but not those that act upon its alanine residue.

It has been reported that DiHPhe chemically synthesized (5) or isolated from a streptomycete (21, 22) was an effective antagonist of phenylalanine for the growth of some bacteria, fungi and young rats. However, the mechanism of this growth inhibition has not been elucidated. Though the structural similarity between DiHPhe and tryptophan is not clearly explained, the present results show that DiHPhe may inhibit the growth not only as an antagonist of phenylalanine but also as that of tryptophan.

Various inhibitors of tryptophan 2,3-dioxygenase have been described previously, such as tryptophan derivatives (competitive), catechol, hydroquinone, p-quinone, L-dihydroxy-phenylalanine and L-epinephrine (non-competitive) (23). However, an inhibitor of indoleamine 2,3-dioxygenase has not been reported. DiHPhe may be a useful tool for unraveling the reaction mechanism and physiological role of this enzyme.

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