Biochemical Pharmacology, Vol. 41, No. 2, pp. 199–203, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITION OF TRYPTOPHAN HYDROXYLASE BY FOOD-DERIVED CARCINOGENIC HETEROCYCLIC AMINES, 3-AMINO-1-METHYL-5*H*-PYRIDO[4,3-*b*]INDOLE AND 3-AMINO-1,4-DIMETHYL-5*H*-PYRIDO[4,3-*b*]INDOLE

MAKOTO NAOI,* SYUN HOSODA,† MIYUKI OTA,‡ TSUTOMU TAKAHASHI§ and TOSHIHARU NAGATSU

Department of Biochemistry, Nagoya University School of Medicine, Nagoya; † Aichi Cancer Center Research Institute, Nagoya; ‡ Department of Psychiatry, Nagoya City University School of Medicine, Nagoya; and § Department of Food and Nutrition, Konan Women's College, Konan, Japan

(*Received* 3 *May* 1990; *accepted* 12 *August* 1990)

Abstract—Food-derived and carcinogenic heterocyclic amines, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), were found to inhibit the activity of tryptophan hydroxylase (TPH) prepared from serotonin-producing murine mastocytomas, P-815 cells. Inhibition of TPH by Trp-P-2 was found to be competitive with the substrate L-tryptophan and non-competitive with the cofactor (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin. The inhibition proved to be reversible; by dialyzing the sample incubated with Trp-P-2, the enzyme activity could be fully recovered. Among a series of heterocyclic amines examined, Trp-P-1, Trp-P-2 and some other heterocyclic amines inhibited TPH activity. Trp-P-2 and other heterocyclic amines were the newly discovered naturally occurring inhibitors of the indoleamine metabolism.

3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and other heterocyclic amines are produced by cooking as products of tryptophan pyrolysis [1], and are carcinogenic and mutagenic [2]. Recently, these amines were found by *in vivo* and *in vitro* experiments [3] to inhibit catecholamine metabolism. They inhibit the enzyme activities of tyrosine hydroxylase [tyrosine tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH] in rat nigro-striatal slices [3] and monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO] in human brain synaptosomal mitochondria [4]. Culture with these amines reduces the enzyme activities of TH and aromatic L-amino acid decarboxylase (aromatic L-amino-acid carboxylyase, EC 4.1.1.28, AADC) in rat clonal pheochromocytoma PC12h cells [5]. Their chemical structures and their origin as pyrolysis products from tryptophan suggest that they may affect the enzymes related to indoleamine metabolism. To examine the effect on the activity of tryptophan hydroxylase [L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4, TPH], the rate-limiting enzyme in serotonin biosynthesis, mastocytoma P-815 cells were used as an enzyme source. The cells are known to produce serotonin and to have a high TPH activity [6].

This paper reports the inhibition of TPH activity by Trp-P-2 and other heterocyclic amines. The inhibition is discussed in relation to the possible role of these amines as food-derived inhibitors of indoleamine metabolism.

MATERIALS AND METHODS

The murine mastocytoma P-815 line, established in DBA mice by Dunn and Potter [7], has been carried in DBA/2 mice [8]. The ascitic fluid was taken, and the cells were gathered by centrifugation at 1000 *g* for 10 min. The cells were washed twice with Hanks' balanced salt solution without serum, and then suspended in the Hanks' solution without serum and stored at −80° until used. The cells (250 mg protein) were diluted with ten times the volume of the extraction medium; 50 mM Tris–acetate buffer, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 5 μg/mL of each antipain, pepstatin, chymostatin, and leupeptin. The cell suspension was homogenized with a Potter–Elvehjem homogenizer and centrifuged at 100,000 *g* for 60 min. The supernatant was passed through a Sephadex G-25 column (2 cm i.d. × 15 cm), which was equilibrated and eluted with 10 mM Tris–acetate buffer, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride. The fractions eluted in the void volume were gathered and used as the enzyme sample. Trp-P-2 and Trp-P-1 were purchased from Wako, and the other heterocyclic amines were donated by Dr. Takashi Sugimura, National Cancer Center Research Institute, Tokyo, Japan. L-Tryptophan and NSD-1015 (*m*-hydroxybenzyl hydrazine) were purchased from Sigma, and dithiothreitol and β-mercaptoethanol, from Nacalai tesque. (6*R*)-L-*erythro*-5, 6, 7, 8-Tetrahydrobiopterin hydrochloride [(6*R*)BH_4] was synthesized according to the method of Muramatsu *et al.* [9]. Catalase prepared from bovine liver was purchased from Boehringer. Organic solvents were of the grade

* To whom correspondence should be addressed.

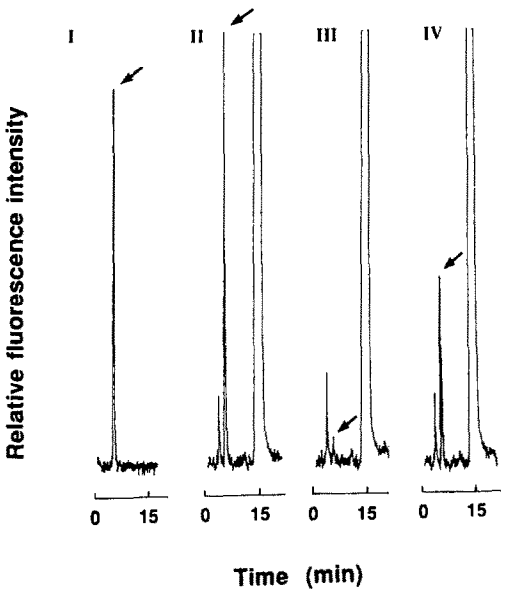

Fig. 1. HPLC patterns of standard 5-HTP and samples prepared from mastocytoma cells. The sample was incubated with 100 μM L-tryptophan in the presence of 100 μM (6*R*)BH_4 (II), or in its absence (III), and treated as described under Materials and Methods. The enzyme activity also was measured in the presence of 100 μM Trp-P-1 (IV). I: standard 5-HTP (5 pmol).

needed for high-performance liquid chromatography (HPLC).

TPH activity was measured by quantitative analysis of 5-hydroxytryptophan (5-HTP) produced from L-tryptophan in the presence of NSD-1015, an inhibitor of aromatic L-amino acid decarboxylase, according to a previously reported method [10] with a slight modification. The enzyme sample (10–50 μg protein) was incubated at 37° for 20 min with 1 mM L-tryptophan in 100 μL of 100 mM HEPES-NaOH buffer, pH 7.6, containing 20 μM (6*R*)BH_4, 1 mM β-mercaptoethanol, catalase (0.8 μg protein) and 0.5 mM NSD-1015. The reaction was terminated by addition of 10 μL of 60% perchloric acid, and the sample was mixed, centrifuged at 15,000 *g* for 10 min, and filtered through a Millex HV filter (pore size, 0.45 μm).

For quantitation of 5-HTP, the sample was applied onto a Shimadzu HPLC apparatus, LC5A, connected to a Shimadzu fluorescence detector, FD-500. The column used was a pre-packed reversed-phase column, ODS-H (4 mm i.d. × 150 mm, Shimadzu Techno-Research, Kyoto, Japan), and the mobile phase was 90 mM sodium acetate–35 mM citric acid buffer, pH 4.35, containing 130 μM disodium EDTA and 2 mM sodium octanesulfonate, to which methanol was added to 11%. The flow rate was 0.8 mL/min. The fluorescence intensity at 345 nm was measured with excitation at 295 nm. Quantitation of 5-HTP was carried out by comparison of the peak area with that of a standard.

To examine the reversibility of the inhibition, Trp-P-2 (1 mM in the final concentration) was incubated with the enzyme sample (3 mg protein) in 2 mL of 10 mM Tris–HCl buffer, pH 7.4, at 37° for 20 min, and then dialyzed against 2 L of 10 mM potassium phosphate buffer, pH 7.4, at 4° overnight. Next, the enzyme activity was measured with a 100 μM concentration of (6*R*)BH_4 and of L-tryptophan. For detection of Trp-P-2 in the sample, an aliquot of the sample (100 μL) was mixed with 10 μL of 60% perchloric acid containing 0.5% ascorbic acid and disodium EDTA, centrifuged at 15,000 *g* for 10 min, and filtered through an HV filter. The sample was applied on the same HPLC apparatus equipped with a fluorescence detector as used for the assay of TPH activity. The column used was an AsahipaK ODP-50 (6 mm i.d. × 250 mm), and the mobile phase was a mixture of 0.1% trifluoroacetic acid and acetonitrile (2:1, v/v). The flow rate was 0.5 mL/min. The fluorescence intensity at 400 nm was measured with excitation at 260 nm. Protein concentration was measured according to Bradford [11], using bovine γ-globulin as standard.

RESULTS AND DISCUSSION

After incubation of the enzyme sample, the 5-HTP produced was detected by HPLC. Figure 1 shows the HPLC pattern of standard 5-HTP (Fig. 1, I) and the samples, the activities of which were measured in the presence and in the absence of (6*R*)BH_4 (Fig. 1, II and III). The data show that TPH activity was dependent on (6*R*)BH_4 being added to the reaction mixture. The kinetic properties of TPH used in these experiments are summarized in Table 1. In terms of the cofactor, (6*R*)BH_4, the

Table 1. Kinetic properties of TPH sample

Substrate	K_m (μM)	V_{max} (pmol/min/mg protein)		
L-Tryptophan*	6.42 ± 0.47	43.9 ± 1.1		
Cofactor	K_{m1} (μM)	V_{max1} (pmol/min/mg protein)	K_{m2} (μM)	V_{max2} (pmol/min/mg protein)
(6*R*)BH_4†	5.22 ± 0.87	24.9 ± 2.9	588 ± 21	41.2 ± 10.2

Each value is the mean ± SD of triplicate measurements of three experiments.
* Enzyme activity was measured in the presence of 1 mM (6*R*)BH_4.
† Enzyme activity was measured with 100 μM L-tryptophan.

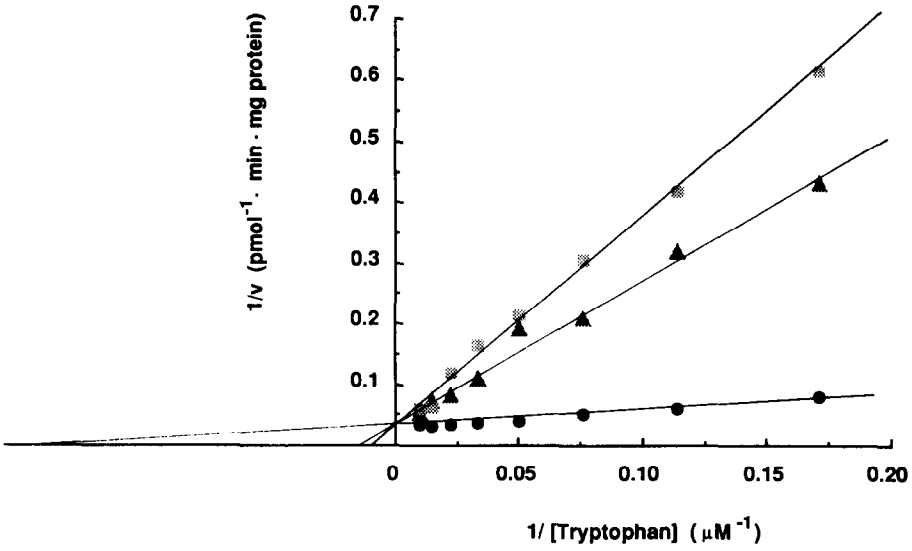

Fig. 2. Effects of the concentration of L-tryptophan on TPH activity in the absence and presence of Trp-P-1 and Trp-P-2. TPH activity was measured with eight different concentrations of L-tryptophan and 1 mM (6*R*)BH_4. The reciprocal of the reaction velocity was plotted against that of the concentration, according to Lineweaver and Burk. Circles represent control, and triangles and squares show the activity measured with 1 mM Trp-P-1 and Trp-P-2 respectively.

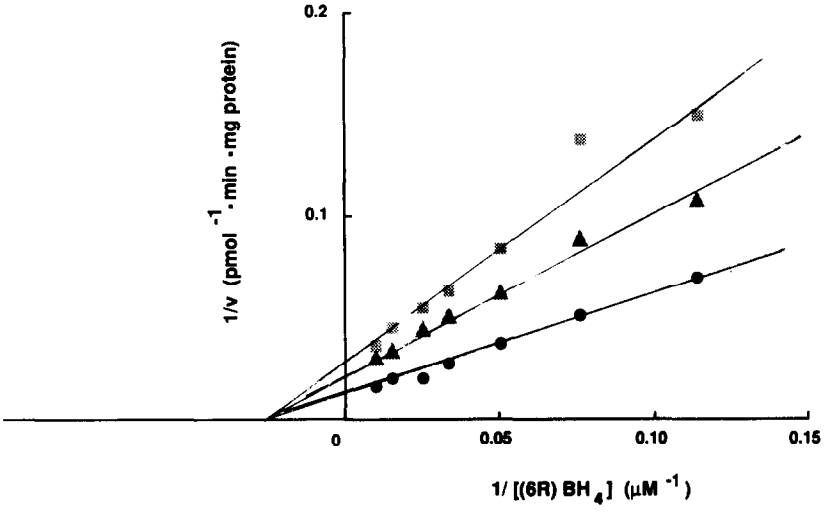

Fig. 3. Effects of the concentration of (6*R*)BH_4 on TPH activity in the absence and presence of Trp-P-1 and Trp-P-2. TPH activity was measured with seven different concentrations of (6*R*)BH_4 and 100 μM L-tryptophan. The reciprocal of the reaction velocity was plotted against that of (6*R*)BH_4 concentration. Circles represent control, and triangles and squares show the activity measured in the presence of 100 μM Trp-P-1 and Trp-P-2 respectively.

enzyme was found to have two different types of affinity to (6*R*)BH_4, with a K_m value of 5 or 588 μM.

As shown in Fig. 1, IV, Trp-P-1 inhibited TPH activity. The effects of Trp-P-2 and Trp-P-1 on TPH activity were examined with various concentrations of a substrate and of (6*R*)BH_4. As shown in Fig. 2, Trp-P-2 and Trp-P-1 inhibited the activity in competition with L-tryptophan. The inhibition constant, K_i, values of Trp-P-2 and Trp-P-1 were 41.7 ± 5.2 and 99.6 ± 12.9 μM (mean ± SD of triplicate measurements of three experiments) respectively. Figure 3 shows that Trp-P-2 and Trp-P-1 inhibited TPH activity measured with various concentrations of (6*R*)BH_4, and the inhibition was non-competitive with the cofactor. The K_i values were 85.7 ± 4.6 and 151 ± 28 μM respectively. The inhibition of TPH by Trp-P-2 was reversible: after incubation of the enzyme sample with or without 1 mM Trp-P-2 for 20 min at 37°, the control and sample were dialyzed to remove Trp-P-2. Before the dialysis, the enzyme activity was reduced by incubation with Trp-P-2 from 49.8 ± 8.5 pmol/min/mg protein to 12.2 ± 5.2 pmol/min/mg protein. After dialysis, the activities of the sample and the control were almost the same: 32.2 ± 8.1 and 29.6 ± 2.9 pmol/min/mg protein, respectively. Using

Table 2. Effects of heterocyclic amines on TPH activity

Compounds	Relative enzyme activity (%)
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2)	57.2 ± 6.9*
3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)	71.9 ± 6.2*
2-Amino-9*H*-pyrido[2,3-*b*]indole	90.9 ± 2.1†
2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole	91.3 ± 2.1†
2-Amino-6-methyldipyrido[1,2-*a*:3′2′-*d*]imidazole	88.0 ± 7.3†
2-Aminodipyrido[1,2-*a*:3′2′-*d*]imidazole	91.3 ± 2.9†
2-Amino-3-methylimidazo[4,5-*f*]quinoline	95.8 ± 4.2
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline	102.2 ± 1.9
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline	90.9 ± 8.8
2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	88.2 ± 6.3†
2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline	78.9 ± 1.2*
(−)-(1*S*, 3*S*)-1-Methyl-1,2,3,4-tetrahydrocarboline-3-carboxylic acid	99.0 ± 10.1
2-Amino-5-phenylpyridine	98.0 ± 4.9
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine	94.1 ± 3.2†

Enzyme activity was measured in the presence of 100 μM heterocyclic amine, using 10 μM L-tryptophan and 1 mM (6*R*)BH_4. Control activity was 26.6 ± 0.8 pmol/min/mg protein. Each value is the mean ± SD of triplicate measurements of four experiments.

*† Statistical significance (Student's *t*-test) for the samples compared to control values is shown as * $P < 0.01$, and † $P < 0.05$.

HPLC-fluorometric detection, Trp-P-2 was not found in the sample after dialysis, even though the sensitivity of the assay was high enough to detect 10 fmol of Trp-P-2.

The effects of a series of heterocyclic amines on TPH activity are summarized in Table 2. The most potent inhibitor was Trp-P-2, followed by Trp-P-1. The compounds with an indole, imidazole, or quinoxaline group inhibited the enzyme activity, whereas those with a quinoline or pyridine group or β-carboline derivative did not affect the enzyme activity.

The data presented in this paper show that TPH was inhibited by Trp-P-2 and Trp-P-1 and that the inhibition was competitive with the substrate, L-tryptophan and non-competitive with the cofactor, (6*R*)BH_4. Trp-P-1 and Trp-P-2 were found to inhibit type A MAO in competition with the substrate [3, 4] and to inhibit AADC in competition with a cofactor, pyridoxal-5-phosphate [12]. The K_i value of type A MAO to Trp-P-2 was the smallest, 1.76 μM, and that of AADC was rather high, 163 μM. The reactions catalyzed by TPH, AADC, and MAO are quite different: hydroxylation of the indole ring, decarboxylation from the amino acid, and oxidative deamination of the amine. The binding site of Trp-P-2 to the active site of these enzymes may also be different. In the case of MAO, the binding site is considered to be the 3-amino group [4]; the binding site of Trp-P-2 to AADC has not been clarified. The data on the relation of the chemical structure and the inhibition of TPH activity indicate that the presence of an indole, imidazole, and quinoxaline group is essential for the inhibition of the TPH activity. The 5*H*-pyrido[2,3-*b*]indole structure seems to be more favourable to binding to the enzyme than 9*H*-pyrido[2,3-*b*]imidazole derivatives. The presence of a methyl group(s) at the 1 and 4 positions seems to increase the affinity of the amines to the enzyme.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a well-known neurotoxin, which inhibits *in vivo* and *in vitro* tyrosine hydroxylation [13], and it elicits the neurodegeneration of dopaminergic neurons in the nigro-striatum in humans [14]. The effect of MPTP on TPH activity has not been reported. Trp-P-2 and other heterocyclic amines inhibit tyrosine hydroxylation in slices of the rat striatum [3], but the mechanism of the inhibition has not been elucidated. On the other hand, Trp-P-2 and Trp-P-1 have been found to inhibit TPH, as reported here, AADC [11], and type A MAO, which oxidizes serotonin [3, 4]. In addition, these amines have been detected in human plasma [15], Trp-P-1 was reported to be transported into the brain through the blood–brain barrier [16], and they are taken up into dopaminergic cells by the transport system specific for dopamine [17]. The transport of these amines is inhibited more markedly by serotonin than by dopamine [17], which suggests that they may be transported by the transport system of serotonin. If such a transport of Trp-P-2 and Trp-P-1 functions in the human brain, these amines may be transported into serotonergic cells and accumulated there; then they may perturb the indoleamine metabolism by inhibiting the enzymes related to their metabolism. Trp-P-2 and Trp-P-1 may be serotonergic neurotoxins comparable to MPTP, a dopaminergic neurotoxin. On the other hand, the data on the inhibition of serotonin biosynthesis by these compounds suggest that they may be involved in regulating the serotonin levels in blood and peripheral cells where serotonin is believed to modulate various immune processes. Thus, these cancer initiators and promoters could possibly play a role in immunologic modulation-related carcinogenesis.

Acknowledgements—This work was supported by a Grant-In-Aid for Scientific Research on Primary Areas, Ministry of Education, Science and Culture, Japan. We thank Dr. Takashi Sugimura, National Cancer Center Research

Institute, Tokyo, Japan, for his supply of heterocyclic amines, and Ms. Kumi Kato for her technical assistance.

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