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PROPERTIES OF TRYPTOPHAN HYDROXYLASE FROM HUMAN CARCINOID TUMOR

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Summary

Tryptophan 5-hydroxylase (L-tryptophan, tetra-hydropteridine: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4) was purified 6-fold from a human carcinoid tumor containing a large amount of 5-hydroxytryptophan as well as 5-hydroxytryptamine. The enzyme was activated following anaerobic dialysis in the presence of 2-mercaptoethanol, and did not require 2-mercaptoethanol and Fe^{2+} for full activity, though these reducing agents shifted the pH optimum for maximum enzyme activity on the more acidic side.

The K_m values were 13 μM for tryptophan in the presence of 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine, 1.2% and 7.1% for oxygen in the presence of either 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine or 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine, respectively, and 50 μM for 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine and 105 μM for 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine. The substrate inhibition by tryptophan was observed with 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine, but not with 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine, and high oxygen levels did not inhibit the hydroxylation in the presence of these reduced pterins.

It was found that *o*-phenanthroline was also a potent inhibitor of tryptophan hydroxylase from this tumor similar to the mast-cell enzyme and the other pterin-dependent aromatic amino acid hydroxylases. The inhibition by this metal chelating agent was competitive with respect to both tryptophan and 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine, but not to molecular oxygen under the assay conditions employed.

The results obtained support the idea that tryptophan hydroxylase found in some human carcinoids appears to function in the same manner as do most other mammalian hydroxylases.

Abbreviations: $MePteH_4$, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine; Me_2PteH_4, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine; Trp(5OH), 5-hydroxytryptophan; NSD 1055, *m*-hydroxy-*p*-bromobenzyloxyamine.

Introduction

In man, the hydroxylation of tryptophan was first demonstrated using slices prepared from the liver metastases of the patients with the carcinoid syndrome [1]. Subsequently, it was found that the homogenate [2] and ammonium sulfate precipitate [3] from the tumor also hydroxylate tryptophan in the presence of 2-mercaptoethanol and a reduced pteridine cofactor, although no detailed kinetic studies of this reaction have been done before the work reported here.

Since the carcinoid tissue from a liver metastasis of a patient accompanied by typical carcinoid syndrome was available in this laboratory, an attempt was made to prepare a cell-free preparation which would hydroxylate tryptophan. This work presents the characteristics of a partially purified tryptophan 5-hydroxylase (L-tryptophan, tetra-hydropteridine: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4) from this tumor.

Materials and Methods

Materials

The carcinoid tissue was a liver metastasis taken within 1 h of death from a patient with the carcinoid syndrome and stored for 4 years at −80° C. L-tryptophan was purchased from Tanabe Amino Acid Fund, $MePteH_4$ and Me_2PteH_4 from Calbiochem, L-5-hydroxytryptophan from Sigma, and beef liver catalase with 50 000 units of specific activity from Boehringer, the mixtures of oxygen and nitrogen gas, and other chemicals from commercial sources. NSD 1055 and pargyline hydrochloride were generous gifts from Dr. Toshiharu Nagatsu, Aichi Gakuin University.

Enzyme assays

The hydroxylation of tryptophan was carried out by shaking 1 ml of reaction mixture in a tube in a 37° C water bath, as described in the previous paper [4,5]. The standard assay solution contained 100 μmol of Tris/acetate buffer (pH 7.6), 200 μg of catalase, 0.2 μmol of $MePteH_4$, 0.7 to 0.8 mg of enzyme and 0.2 μmol of L-tryptophan. The last two additions were tetra-hydropterin and L-tryptophan in that order.

When the effect of pH on tryptophan hydroxylation was examined, the pH values in the assay mixture containing 100 μmol of Tris/acetate buffer at varying pH were checked by the pH meter. After a 10 min incubation, the reaction was stopped by adding 0.3 ml of 20% perchloric acid. The mixture was centrifuged for 10 min at 10 000 × *g*, and the supernatant fraction was directly determined fluorometrically for 5-hydroxytryptophan, since the preliminary experiment revealed that any fraction from the present tumor formed neither 5-hydroxytryptamine nor 5-hydroxyindoleacetic acid. Both were determined fluorometrically after the extraction from purified butanol or peroxide-free ether, respectively [6]. Either 0.5 mM NSD 1055 or 0.4 mM pargyline hydrochloride (*N*-benzyl-*N*-methyl-2-propynylamine hydrochloride) did not affect the rate of hydroxylation. It was also found that the fluorescent curve and intensity of product in the supernatant accorded well with those of authentic 5-hydroxy-

tryptophan obtained according to the fluorometric method for tissue 5-hydroxytryptophan [7,8]. 1 ml of supernatant was added to 0.3 ml of concentrated hydrochloric acid and the fluorescence due to 5-hydroxytryptophan was measured (295 nm activation, 540 nm fluorescence). The protein concentrations were determined by the Lowry-Folin method [9].

Results

Clinicopathologic outlines of a patient with carcinoid tumor

A 65-year-old Japanese man complained of discomfort in the epigastrium and repeated facial flushing in 1971. In 1972, severe diarrhea appeared with rapid enlargement of the liver, and he died of general emaciation 18 months after the onset of the disease. The urinary excretion of 5-hydroxyindoleacetic acid in the patient was 42—90 mg in 24 h. An autopsy revealed multiple metastases in liver, bones, lymph nodes and kidneys, and eventually a small primary tumor measuring 20 × 15 mm was found in the left upper bronchus. The tumor nodules, either primary or metastatic, revealed the same histologic pictures characteristic of carcinoid. The tumor cells gave a positive argyrophil reaction but were argentaffinnegative. A solid metastatic nodule with less necrosis removed from the liver contained 49.5 μg of 5-hydroxytryptophan, 106.5 μg of 5-hydroxytryptamine and 11.6 μg of 5-hydroxyindoleacetic acid per g of wet tissue by the fluorometric method [6].

Partial purification of tryptophan hydroxylase

All procedures were carried out below 4° C. The frozen tumor tissues (usually 4 g as wet tissue) were cut into small pieces with a surgical blade and added to 10 ml of Hanks' solution [10] (without phenol-red, glucose and bovine serum albumin, pH 7.2), containing 14.2 mM 2-mercaptoethanol, and homogenized in Potter's homogenizer. The homogenate was centrifuged at 9500 × g for 10 min and the supernatant was centrifuged at 135 000 × g for 60 min. Powdered ammonium sulfate was added to the final supernatant until 0.25 saturation was attained. The precipitate was removed by centrifugation and additional ammonium sulfate was added to the supernatant until 0.4 saturation was attained. The precipitate was dissolved in 4 ml of 10 mM Tris · HCl buffer (pH 7.4), containing 14.2 mM 2-mercaptoethanol, and dialyzed in a cellophan tube at 4° C for 24 h against 1 litre of the same buffer solution with two changes of external fluid after nitrogen was bubbled enough through the latter for anaerobiosis.

The analytical details of a typical purification are shown in Table I. The enzyme is purified 6-fold by this simple purification step with a yield of about 80%. The specific activity of most purified enzyme was found to be 14 times higher than that of crude enzyme from carcinoid tumor reported by others [2]. The enzyme was stored under nitrogen at 4° C with no loss of enzyme activity for a period of two months, but when stored under air, 73% of activity was lost within 24 h and more than 90% within 48 h.

Characteristics of tryptophan hydroxylase

Using a partially purified enzyme preparation, we could not find the

TABLE I

PURIFICATION OF TRYPTOPHAN HYDROXYLASE FROM CARCINOID TUMOR

The reaction was carried out using both 0.2 μmol of tryptophan and $MePteH_4$ under the same conditions as described in a text. Each fraction was used after dialyzed in the same manner to ammonium sulfate precipitate (25—40%).

Fraction	Volume (ml)	Total protein (mg)	Specific activity (nmol/mg protein/min)	Purification (-fold)	Recovery (%)
Homogenate	13	390	0.009	1	100
135 000 × g supernatant	10	92.5	0.034	3.8	90
Ammonium sulfate					
0—25%	2	12.9	0.008	—	—
25—40%	4	54.4	0.054	6.0	83
40—60%	2	28.0	0.018	—	—

absolute requirement of 2-mercaptoethanol for tryptophan hydroxylation, though this reducing agent was essential for the activity of tryptophan hydroxylase from neoplastic mast cells [4,5]. The formation of 5-hydroxytryptophan was virtually proportional to protein concentration from 0.2 mg to 5 mg. As shown in Fig. 1, however, the rate of tryptophan hydroxylation decreased remarkably with time, and when used with Me_2PteH_4, the hydroxylation ceased completely after 30 min. Fig. 1 also illustrates that the hydroxylation with $MePteH_4$ was 1.5 times higher than that with Me_2PteH_4 in an initial 10 min incubation. In earlier observations [5], we showed that pH optimum for full activity of tryptophan hydroxylase purified from neoplastic mast cells was lowered either by increasing 2-mercaptoethanol concentration or by adding Fe^{2+}. Tryptophan hydroxylase from carcinoid also had the pH optimum at 7.6 in the absence of 2-mercaptoethanol, and the peak of activity deviated to pH 7.2 in the presence of 35.5 mM 2-mercaptoethanol. By adding 0.2 mM Fe^{2+} to the above assay systems, the pH-activity curves shifted to a more acidic region with

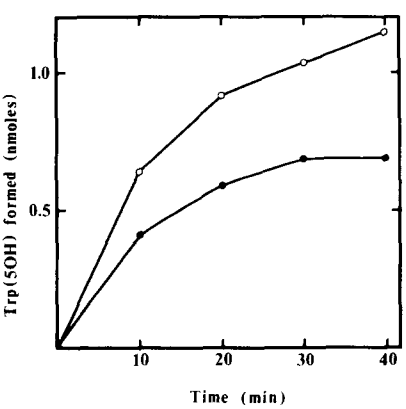

Fig. 1. Rate of hydroxylation of tryptophan with the use of $MePteH_4$ (○) or Me_2PteH_4 (●). The reaction was carried out using both 0.2 μmol of tryptophan and tetra-hydropterin, and 0.78 mg of enzyme protein at pH 7.6, as described in a text.

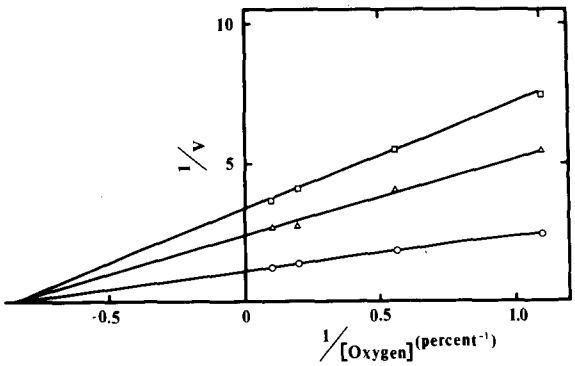

Fig. 2. Double-reciprocal plots against oxygen concentration at several fixed concentrations of tryptophan. The reaction was carried out using 0.2 μmol of $MePteH_4$ and 0.75 mg of enzyme protein. The concentrations of tryptophan used were: □, 0.005 mM; △, 0.01 mM; and ○, 0.2 mM.

the peaks of activity at pH 6.8 in the absence of 2-mercaptoethanol and pH 6.4 in its presence, and exhibited shoulders of enzyme activity around pH 7.2 and 7.6, respectively, each corresponding to pH optimum for full activity under the original assay conditions without added Fe^{2+}.

Kinetic studies of tryptophan hydroxylase

In view of the fact that 2-mercaptoethanol and Fe^{2+} did not affect significantly the rate of hydroxylation at optimum pH by tryptophan hydroxylase from the present carcinoid tumor, the kinetic studies were carried out under simple assay conditions without these substances. In double-reciprocal plots of velocity against $MePteH_4$ or Me_2PteH_4 concentrations at 0.2 mM tryptophan, the apparent Michaelis constant (K_m) was 50 μM for $MePteH_4$ and 105 μM for Me_2PteH_4. The V with $MePteH_4$ was 1.6 times higher than that with Me_2PteH_4. The double-reciprocal plots of velocity against oxygen concentration for a fixed concentration of $MePteH_4$ or Me_2PteH_4 showed that the K_m for oxygen was 1.2% with 0.2 mM $MePteH_4$ and 7.1% with 0.4 mM Me_2PteH_4. There was no inhibition of the reaction by high oxygen levels. Fig. 2 shows double-recip-

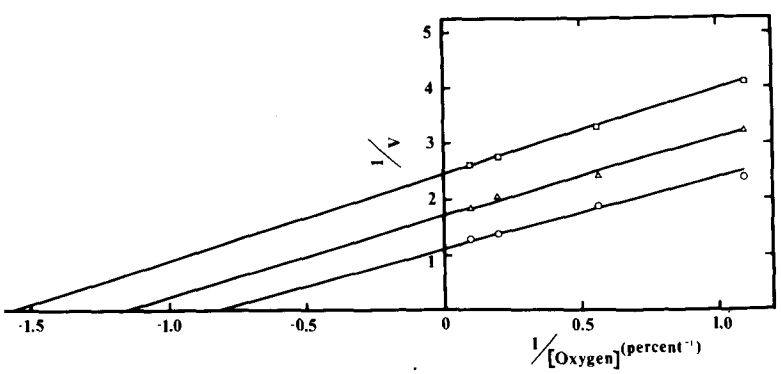

Fig. 3. Double-reciprocal plots against oxygen concentration at several fixed concentrations of $MePteH_4$. The reaction was carried out using 0.2 μmol of tryptophan and 0.75 mg of enzyme protein. The concentrations of $MePteH_4$ used were: □, 0.025 mM; △, 0.05 mM; and ○, 0.2 mM.

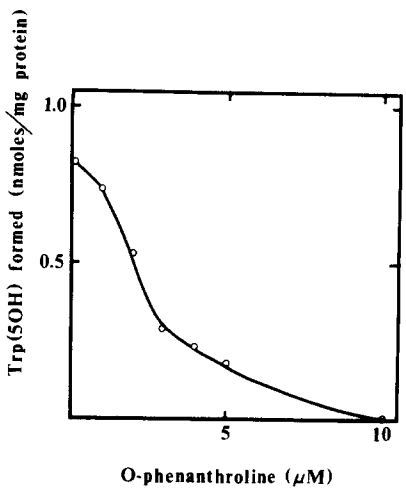

Fig. 4. Inhibition of tryptophan hydroxylase by o-phenanthroline. The reaction was carried out using both 0.2 μmol of tryptophan and $MePteH_4$, and 0.78 mg of enzyme protein. Other experimental conditions are given in a text.

rocal plots of velocity against oxygen concentration for different fixed concentrations of tryptophan. The lines obtained converged on the horizontal axis. On the other hand, when the $MePteH_4$ concentrations were varied, the lines in the $1/v$ versus $1/O_2$ plots were nearly parallel, as shown in Fig. 3.

Kinetic studies on inhibition of tryptophan hydroxylase by o-phenanthroline

The previous studies disclosed that *o*-phenanthroline was a potent inhibitor of tryptophan hydroxylase purified from neoplastic mast cells, and the inhibition by this substance was competitive with respect to both tryptophan and Me_2PteH_4, but not to molecular oxygen [5]. As shown in Fig. 4, 50% inhibition of hydroxylation was observed at 2.4 μM *o*-phenanthroline which was

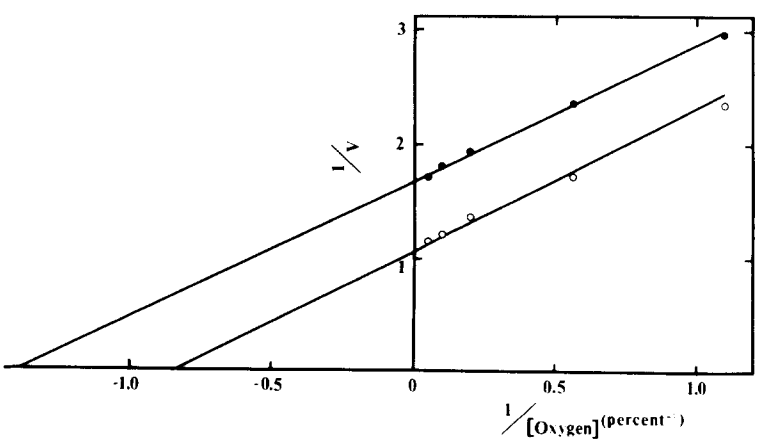

Fig. 5. Double-reciprocal plots against oxygen concentration in the presence (●) and absence (○) of o-phenanthroline. The experimental conditions are the same as in Fig. 4, except that 0.75 mg of enzyme protein and 2 nmol of *o*-phenanthroline were used.

close to 2.5 μM in the mast cell hydroxylase [5]. The double-reciprocal plots of velocity against tryptophan concentration in the presence and absence of 2 μM *o*-phenanthroline showed that the inhibition by *o*-phenanthroline was competitive with respect to tryptophan and K_i was found to be 0.82 μM. The apparent K_m for tryptophan was 13 μM, and significant substrate inhibition was seen with this hydroxylase, when the tryptophan concentration was increased in the presence of $MePteH_4$. $MePteH_4$ also counteracted the *o*-phenanthroline inhibition which proved to be competitive with respect to $MePteH_4$ added at $1.25 \cdot 10^{-5}$ to $1.5 \cdot 10^{-4}$ M. K_i was calculated to be 2.6 μM. As shown in Fig. 5, however, in $1/v$ versus $1/O_2$ plots with and without added *o*-phenanthroline the lines obtained were almost parallel.

Discussion

In recent years the kinetic studies of tryptophan hydroxylases have been achieved in several mammalian tissues such as brain from rat [11] and rabbit [7,12], pineal gland from rat [11] and beef [13], and neoplastic mast cells from mouse [5], and in chromobacterium violaceum [8]. It is a wonder that no detailed descriptions of tryptophan hydroxylase from human carcinoid tumors have been made thus far, since it is now well established that 5-hydroxytryptamine, a direct decarboxylation product of 5-hydroxytryptophan, is actually synthesized by the tumor tissues from patients accompanied by the carcinoid syndrome. The difficulty for obtaining ample amounts of fresh tumor tissues might have hampered the analyses. It seems more probable to us, however, that the kinetic studies of tryptophan hydroxylase from carcinoid tumors were limited by the relatively low hydroxylase activity found in the tumors [2,3].

In the present study, we showed that the tumor tissues examined contained not only large amounts of 5-hydroxytryptamine, but also its immediate precursor 5-hydroxytryptophan, at about half the level of the former, indicating that in the biosynthesis of 5-hydroxytryptamine from tryptophan by this tumor, the step of decarboxylation rather than hydroxylation is rate-limiting. The failure to detect the formation of 5-hydroxytryptamine in our assay systems using any fraction from the tumor also supports the relatively weak activity of aromatic amino acid decarboxylase, compared to the marked activity of tryptophan hydroxylase. In addition, the reasonable activity of tryptophan hydroxylase was fluorometrically detectable only following anaerobic dialysis of each tumor fraction in the presence of 2-mercaptoethanol in spite of the long storage of the frozen tissues (4 years). It should be noted here, however, that the dialysis and storage of enzyme under air caused a marked loss of enzyme activity and could lead to an erroneous assay of this enzyme. In the initial characterization of tryptophan hydroxylase from one carcinoid tumor, it was found that the enzyme did not require 2-mercaptoethanol for its activity, though the requirement for this reducing agent was absolute in the mast-cell enzyme [4,5]. The addition of either 2-mercaptoethanol or Fe^{2+} lowered pH optimum for full enzyme activity, as observed in the mast-cell enzyme [5]. The kinetic studies showed that the apparent K_m values for tetra-hydropteridines were 105 μM for Me_2PteH_4 and 50 μM for $MePteH_4$. Those were slightly smaller than those reported for tryptophan hydroxylases from the rabbit hindbrain [7] and the

mouse neoplastic mast cells [5]. The apparent K_m for oxygen in the presence of Me_2PteH_4 was 7.1%. This is close to that reported for the mast-cell enzyme [5], and is one-third the value reported for tryptophan hydroxylase from the rabbit brain [7]. When $MePteH_4$ was used as cofactor, the K_m value of the present tryptophan hydroxylase for oxygen was greatly reduced, though no inhibition of the reaction by excess oxygen was observed. Similar observations have also been made in the rabbit-brain enzyme with tetra-hydrobiopterin as cofactor [7]. The K_m for tryptophan was 13 μM with the use of $MePteH_4$ as cofactor. The value is not significantly different from that reported for hydroxylases from beef pineal [13] and mouse neoplastic mast cells [5], regardless of the pterin cofactors used, but is extremely lower than that reported for the enzyme purified from rabbit hindbrain [7]. It has also been reported that when the hydroxylase activity was determined in the presence of Me_2PteH_4, the K_m values of all brain enzymes from rat [11], rabbit [7] and beef [14] for tryptophan were on the order of more 10-fold higher than those described for other mammalian hydroxylases [5,13]. In this respect, tryptophan hydroxylase from the present carcinoid tumor resembles pineal or mast-cell enzyme rather than brain enzyme. The similarity between tryptophan hydroxylases from carcinoid tumor and neoplastic mast cells is also supported by such common kinetic properties as the converging lines on the horizontal axis at different fixed tryptophan concentrations (Fig. 2) and the parallel lines at several fixed concentrations of $MePteH_4$ (Fig. 3), both in the $1/v$ versus $1/O_2$ plots [5].

Furthermore, the kinetic studies on inhibition of the present hydroxylase by *o*-phenanthroline disclosed that the extent and mode of inhibition (50% inhibition at 2.4 μM, competitive with respect to both tryptophan and $MePteH_4$, but not to molecular oxygen, see Fig. 4 and 5), and lower K_i values calculated were very similar to those found in tryptophan hydroxylase from mouse neoplastic mast cells [5]. From all of the results discussed here, it is apparent that tryptophan hydroxylase from human carcinoid tumor shares many of the characteristics of the other mammalian hydroxylases. Thus, the present work adds additional support to the idea that tryptophan hydroxylase in the carcinoid tumor producing 5-hydroxyindoles operates through the same hydroxylating system as do most other mammalian enzymes.

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