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FURTHER STUDIES ON TRYPTOPHAN HYDROXYLASE FROM NEOPLASTIC MURINE MAST CELLS

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Summary

Tryptophan hydroxylase (tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating) EC 1.14.16.4) purified from the neoplastic murine mast cells by hydroxylapatite chromatography following ammonium sulfate fractionation showed maximum activity at pH 6.0 in the presence of 2-mercaptoethanol, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine and Fe^{2+}, and pH 7.6 to 8.0 in the absence of added Fe^{2+}.

The K_m values were 38.5 μM and 22.2 μM for tryptophan, 298 μM and 204 μM for 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetra-hydropteridine, and 6.45% for oxygen in either presence or absence of added Fe^{2+}, respectively.

From kinetic data the reaction mechanism of tryptophan hydroxylation appears to be of the sequential, rather than the ping-pong, type. Tryptophan hydroxylase from mast cells was considerably inhibited by *o*-phenanthroline like phenylalanine hydroxylase as well as tyrosine hydroxylase from other sources, and its K_i was between 1.2 μM and 4.53 μM. It was found that the inhibition by *o*-phenanthroline was competitive with respect to both tryptophan and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, but not molecular oxygen under the assay conditions employed.

Introduction

In mammals, the presence of marked tryptophan hydroxylase activity was first demonstrated in intact murine neoplastic mast cells by Levine et al. [1]. We confirmed their study, and showed that in the biosynthesis of 5-hydroxytryptamine from tryptophan, those cells which contain small but definite amounts of 5-hydroxytryptophan and 5-hydroxytryptamine exhibit marked hydroxylation and slight decarboxylation [2]. Subsequently, certain properties

Abbreviations: Me_2PteH_4, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine; Trp-(5OH), 5-hydroxytryptophan.

of tryptophan hydroxylase partially purified from a microsome-free supernatant fraction of neoplastic mast cells were described [3]. In recent years tryptophan hydroxylases have been studied in a number of mammalian tissues such as carcinoid tumor [4,5], intestine [5,6], pineal gland [4,7—10] and brain [4,7,8,11—14], but have not been obtained in a pure state. Among the different tissues studied, the murine neoplastic mast cells seem to be the richest source of the enzyme found so far [4]. In addition, these cells have the further advantage that the enzyme preparation from these cells was free from 5-hydroxytryptophan decarboxylase due to the lower activity of nonspecific aromatic amino acid decarboxylase in intact tumor cells [3].

In our original paper, we stressed that during the course of purification of tryptophan hydroxylase from the neoplastic mast cells the enzyme activity totally lost on disruption of the cells was largely recovered following anaerobic dialysis of the crude extract in the presence of 2-mercaptoethanol [3]. This mode of purification was also employed in the present study for obtaining the preparation of tryptophan hydroxylase with a higher specific activity from mast cells. The enzyme thus obtained is still too crude for full elucidation of its physical properties, but is suitable for many kinetic studies in much greater detail than has previously been possible.

This paper presents the kinetic studies of the interaction of the enzyme with tryptophan as well as oxygen as substrates and Me_2PteH_4 as cofactor, together with a study of the enzyme inhibition by *o*-phenanthroline as a metal-chelating agent. The results obtained not only give insight into the enzyme mechanism, but also serve as a basis for evaluating the physiological role of the enzyme in the biosynthesis of 5-hydroxytryptamine.

Materials and Methods

Materials

The neoplastic mast cells were obtained from ascitic fluid of DBA mice. The cells were transplanted, harvested and treated as in the previous study [3]. L-Tryptophan was purchased from Tanabe Amino Acid Fund, Me_2PteH_4 from Calbiochem, 5-L-hydroxytryptophan from Sigma, and beef liver catalase with approximately 50 000 units of specific activity and reduced glutathione from Boehringer. Hydroxylapatite was obtained from Bio-Rad, and mixtures of oxygen and nitrogen gas and other chemicals from commercial sources. Glutathione peroxidase was prepared according to the procedure of Nakamura et al. [15].

Enzyme assays

Hydroxylation of tryptophan was carried out by shaking (80 times per min) 1 ml of reaction mixture in a tube (1.5 × 10 cm) in a 37° C water bath. All tubes and reaction mixtures were kept in ice until used. To each tube was added in this order: either Tris/acetate buffer (pH 6.0), 200 μmol; 2-mercaptoethanol, 35.5 μmol; ferrous ammonium sulfate, 0.2 μmol; 100 to 130 μg of enzyme protein, in a total volume of 0.6 ml, or Tris/acetate buffer (pH 8.0); 2-mercaptoethanol; and enzyme, as above, in a total volume of 0.5 ml. The

tubes were preincubated for 5 min at 37° C. Following the prior incubation each tube was equilibrated at 0° C before adding 200 μg of catalase, 0.1 to 0.8 μmol of Me_2PteH_4 and 0.004 to 0.2 μmol of L-tryptophan in that order. After a 12 min incubation, the reaction was stopped by adding 0.3 ml of 20% perchloric acid. The mixture was centrifuged and the supernatant fraction was directly determined fluorometrically for 5-hydroxytryptophan, since the purified enzyme was found to be contaminated with none of nonspecific aromatic amino acid decarboxylase activity. 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 [16].

Results

Purification of tryptophan hydroxylase

All manipulations were performed at 4° C. The neoplastic mast cells (usually 8 ml as a washed cell pellet) were added to 15 ml of ice-cold Hanks' solution [17] (without phenol-red, glucose and bovine serum albumin, pH 7.2), containing 14.2 mM 2-mercaptoethanol, disrupted by freezing-thawing three times and homogenized gently 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 which contained the bulk of enzyme activity was dissolved in 3 ml of 10 mM Tris · HCl buffer (pH 7.4), containing 14.2 mM 2-mercaptoethanol, and dialyzed in a cellophane tube at 4° C for 15 h against 1 litre of the same buffer solution after nitrogen was bubbled enough through the latter for anaerobiosis. The dialyzed enzyme solution was applied to a column, 1 × 12 cm, of hydroxylapatite (5 g) equilibrated with 10 mM potassium phosphate buffer (pH 7.0), containing 14.2 mM 2-mercaptoethanol, and the column was developed with a linear gradient made from

TABLE I

PURIFICATION OF TRYPTOPHAN HYDROXYLASE FROM NEOPLASTIC MAST CELLS

The reaction was carried out using both 0.2 μmol of tryptophan and Me_2PteH_4 under the same conditions as described in a text.

Fraction	Volume (ml)	Total protein (mg)	Specific activity (nmol/mg protein/min)		Purification (-fold)		Recovery (%)	
			pH 6.0	pH 8.0	pH 6.0	pH 8.0	pH 6.0	pH 8.0
Whole homogenate	23.0	816	0.108	0.033	1	1	100	100
135000 × *g* supernatant	17.5	271	0.404	0.059	3.7	1.8	124	60
$(NH_4)_2SO_4$ precipitate (20 to 40%)	4.2	81.9	0.841	0.454	7.8	13.8	78	138
Hydroxylapatite eluate	1.0	10.4	2.27	1.34	21.0	40.6	27	52

200 ml of the equilibration buffer and 200 ml of 0.3 M potassium phosphate buffer (pH 7.0), containing 14.2 mM 2-mercaptoethanol. Fractions of 4 ml each were collected at a flow rate of 20 ml per h. Under these conditions, the enzyme activity was eluted between fractions 45 and 75 with the peak at fraction 59. The enzyme fractions with a higher activity were collected, concentrated by ultrafiltration, and dissolved in 1 ml of 10 mM Tris · HCl buffer (pH 7.4), containing 14.2 mM 2-mercaptoethanol. The final enzyme sample showed faintly brown color having absorbance peaks at 413, 535 and 570 nm, and can be stored at 0° C without no significant loss in enzyme activity for few weeks in a Thunberg tube, in which air was replaced by nitrogen, though the solution became slightly turbid during storage.

The analytical details of a typical purification are shown in Table I. From this table it is noted that an increase in specific activity is more marked in the assay carried out at pH 8.0 without added Fe^{2+}, particularly in the ammonium sulfate fractionation, than in that done at pH 6.0 with added Fe^{2+}. Since the ammonium sulfate fractionation in the case of the pH 8.0 assay resulted in a higher recovery of enzyme activity, it is assumed that the native enzyme before this fractionation may exist largely in an inactive form. This assumption is consistent with the previous study showing that the hydroxylase activity lost totally on disruption of cells is recovered following anaerobic dialysis in the presence of 0.1 M 2-mercaptoethanol [3]. Table I also indicates that Fe^{2+} added at the pH 6.0 assay activates largely the inactive form of enzyme during a 5 min preincubation, since the enzyme activities at the pH 6.0 assay with added Fe^{2+} by whole homogenate and 135 000 × *g* supernatant were, respectively, 3 and 7 times higher than those at the pH 8.0 assay without added Fe^{2+}.

Characteristics of tryptophan hydroxylase

The preliminary experiment using the final enzyme preparation purified through the hydroxylapatite column chromatography confirmed again the essentiality of oxygen, Me_2PteH_4 and 2-mercaptoethanol for hydroxylation, as described in the previous study [3].

It is also found that tryptophan hydroxylase from mast cells became more sensitive to inactivation by hydrogen peroxide generated endogenously as its specific activity increases through the purification steps. As shown in Fig. 1, the rate of tryptophan hydroxylation by most purified enzyme was decreased considerably in the absence of added catalase in the case of the pH 8.0 assay. On the other hand, the decrease in hydroxylation was slight in the presence of Fe^{2+}, probably due to protecting effect of Fe^{2+} on some part of the tryptophan hydroxylation system sensitive to inactivation by hydrogen peroxide like the rabbit hindbrain tryptophan hydroxylase [12]. It was found that 5 μg of glutathione peroxidase with specific activity of 70.4 [15] can replace 200 μg of catalase in protecting the tryptophan hydroxylation system from H_2O_2-mediated inactivation. In Fig. 1, the non-linearity of tryptophan hydroxylation with incubation time in either case of assay at pH 8.0 without added Fe^{2+} or at pH 6.0 with added Fe^{2+} remains unexplained, since a similar phenomenon was constantly observed even when ample amounts of substrate, cofactor and catalase were added to the reaction mixture. Also 5-L-hydroxytryptophan added exogenously on the level with that produced endogenously does not

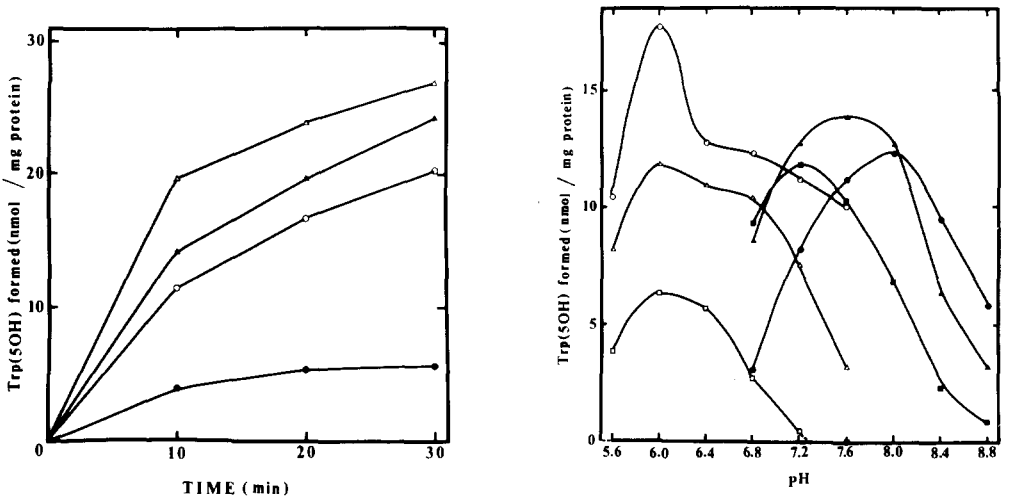

Fig. 1. Rate of hydroxylation of tryptophan in the presence (△, ○) and absence (▲, ●) of catalase. The reaction was carried out at either pH 6.0 with added Fe^{2+} (△, ▲) or pH 8.0 without added Fe^{2+} (○, ●) as described in a text. The amounts of tryptophan, Me_2PteH_4, and enzyme used were 0.2 μmol, 0.2 μmol, and 0.105 mg, respectively.

Fig. 2. Effect of pH on tryptophan hydroxylation with (○, △, □) and without (●, ▲, ■) added Fe^{2+}. Reaction mixture and conditions were the same as in a text, except that 0.1 mg of enzyme protein, both 0.2 μmol of tryptophan and Me_2PteH_4, and varied concentrations of 2-mercaptoethanol (○, ●, 35.5 mM; △, ▲, 71 mM; and □, ■, 142 mM) were used.

inhibit the hydroxylation significantly. Fig. 2 shows the pH-activity curves at varied concentrations of 2-mercaptoethanol in either presence or absence of Fe^{2+}. In the absence of Fe^{2+} and the presence of 35.5 mM 2-mercaptoethanol the pH optimum at 8.0 was found for the tryptophan hydroxylation and no hydroxylation was demonstrable at pH 6.0, as described previously [3]. The peaks of activity deviated to pH 7.8 and 7.6 in the presence of 71 mM and 142 mM 2-mercaptoethanol, respectively. On the other hand, in the presence of Fe^{2+} the pH optimum of activity was constantly 6.0 and the rates of tryptophan hydroxylation were decreased as the concentrations of 2-mercaptoethanol were raised. In the latter case the shoulders of enzyme activity were observed around pH 6.8 in the presence of 35.5 and 71 mM 2-mercaptoethanol. Table II

TABLE II

EFFECT OF GAS PHASES ON TRYPTOPHAN HYDROXYLASE ACTIVITY

Enzyme assay was performed as described in Table I, except that 0.106 mg of enzyme protein was used under the conditions of the indicated gas phases.

Conditions		Hydroxylase activity (nmol/mg protein/min)	
Activation	Incubation	pH 6.0	pH 8.0
Under air	Under air	1.52	0.89
Under air	Under oxygen	0.68	1.07
Under nitrogen	Under air	1.71	1.25
Under nitrogen	Under oxygen	0.82	1.82

reveals the effect of either air or nitrogen in enzyme activation on the tryptophan hydroxylation under the conditions of air and oxygen. In the case of the pH 8.0 assay without added Fe^{2+} the anaerobic preincubation of enzyme containing 35.5 μmol of 2-mercaptoethanol at 37°C for 5 min resulted in higher enzyme activities than those under the aerobic preincubation, whereas in the assay case at pH 6.0 with added Fe^{2+} the formations of 5-hydroxytryptophan were decreased considerably under oxygen regardless of the conditions of activation.

Kinetic studies with and without added Fe^{2+}

As described above, the most prominent feature of mast cell hydroxylase was the deviation of pH optimum to 6.0 in the presence of Fe^{2+}, so that the kinetic studies were carried out by varying the concentrations of the substrates of reaction (tryptophan, Me_2PteH_4, and oxygen) at different fixed concentrations of each substrate in either presence or absence of Fe^{2+}. The double-reciprocal plots of velocity against tryptophan concentration were shown in Fig. 3. The almost parallel lines were obtained and the apparent Michaelis constants (K_m) of tryptophan were increased to 38.5 μM in the presence of Fe^{2+} from 22.2 μM in its absence. Fig. 4 shows double-reciprocal plots of velocity against Me_2PteH_4 concentration for different fixed concentrations of tryptophan. At concentrations of $2 \cdot 10^{-4}$ M tryptophan and $2 \cdot 10^{-4}$ M Me_2PteH_4 the lines obtained are nearly parallel and the K_m for Me_2PteH_4 was found to be 298 μM in the presence of Fe^{2+} and 204 μM in its absence. Under the assay condition without Fe^{2+}, however, when tryptophan concentrations

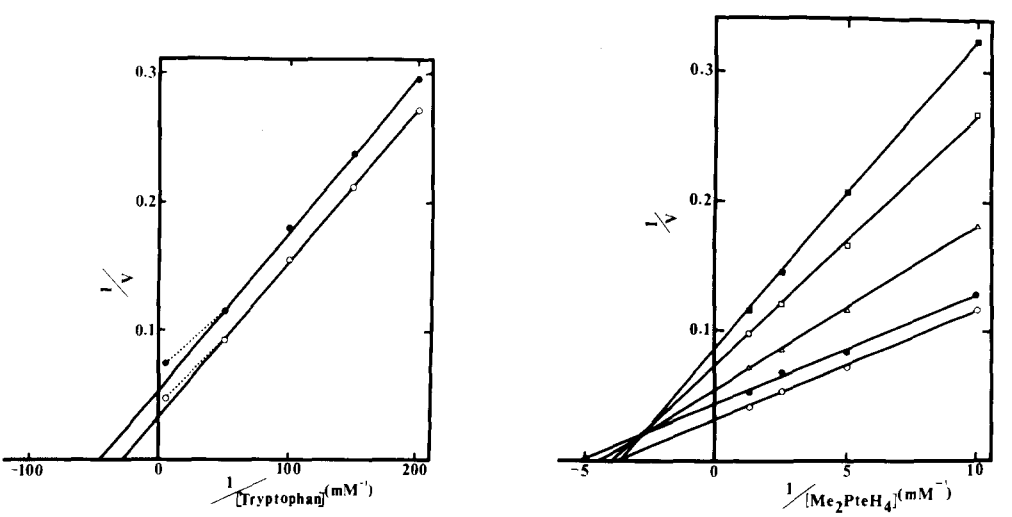

Fig. 3. Double-reciprocal plots against tryptophan concentration in the presence of 0.2 mM Me_2PteH_4 with (○) and without (●) added Fe^{2+}. The enzyme assay was performed at either pH 6.0 (○) or pH 8.0 (●) under air using 0.11 mg of enzyme protein, as given in a text.

Fig. 4. Double-reciprocal plots against Me_2PteH_4 concentration at 0.2 mM tryptophan with (○) and without (●) Fe^{2+}, and at several fixed concentrations of tryptophan in the absence of added Fe^{2+}. The reaction was carried out at either pH 6.0 (○) or pH 8.0 (●, △, □, ■) under air using 0.125 mg of enzyme protein, as described in a text. The concentrations of tryptophan used were: ■, 0.0066 mM; □, 0.01 mM; △, 0.02 mM; and ○, ●, 0.2 mM.

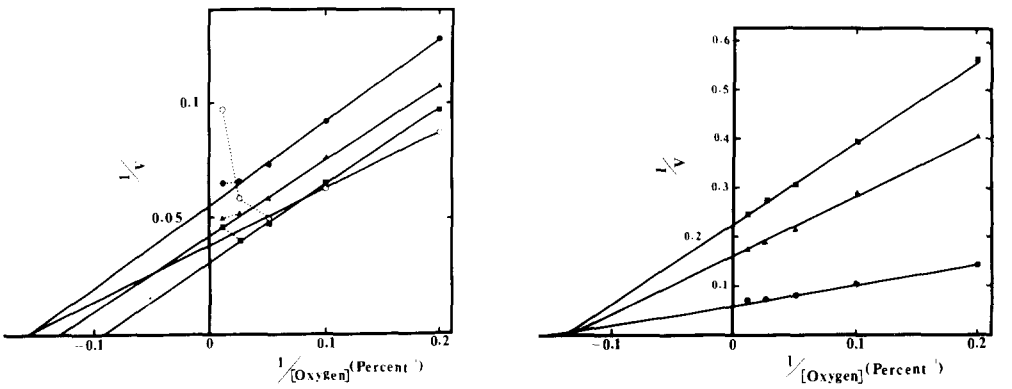

Fig. 5. Double-reciprocal plots against oxygen concentration at 0.2 mM tryptophan with (○) and without (●) Fe^{2+}, and at several fixed concentrations of Me_2PteH_4 in the absence of added Fe^{2+}. The reaction was carried out at either pH 6.0 (○) or pH 8.0 (●, ▲, ■) using 0.11 mg of enzyme protein, as described in a text. The concentrations of Me_2PteH_4 used were: ○, ●, 0.2 mM; ▲, 0.4 mM; and ■, 0.8 mM.

Fig. 6. Double-reciprocal plots against oxygen concentration at several fixed concentrations of tryptophan. The reaction was carried out using 0.2 μmol of Me_2PteH_4 and 0.115 mg of enzyme protein at pH 8.0 without added Fe^{2+}, as given in a text. The concentrations of tryptophan used were: ■, 0.0066 mM; ▲, 0.01 mM; and ●, 0.2 mM.

were decreased until $2 \cdot 10^{-5}$ M, the lines obtained intersected at almost one point to the left of the vertical axis above the horizontal axis. Fig. 5 shows double-reciprocal plots of velocity against oxygen concentration for changing fixed concentrations of Me_2PteH_4. At concentrations of $2 \cdot 10^{-4}$ M Me_2PteH_4 and $2 \cdot 10^{-4}$ M tryptophan the lines obtained converge on the horizontal axis and the K_m for oxygen was 6.45% under both assay conditions. The marked inhibition of hydroxylase at higher oxygen concentration in the presence of Fe^{2+} is consistent with the result mentioned above (Table II). This figure also illustrates that when Me_2PteH_4 concentrations were raised the lines obtained were roughly parallel. On the other hand, when the tryptophan concentrations were varied without added Fe^{2+} the lines in the $1/v$ versus $1/O_2$ plots tended to converge on the horizontal axis, as shown in Fig. 6.

Kinetic studies on inhibition of tryptophan hydroxylase by o-phenanthroline

It has been reported that iron chelators such as 2,2'-dipyridine, *o*-phenanthroline and 8-hydroxyquinoline caused marked inhibition of phenylalanine hydroxylase [19]. Since the extent of inhibition of this enzyme by *o*-phenanthroline was strongest, the present experiment was conducted to examine the effect of this potent inhibitor on tryptophan hydroxylase purified from neoplastic mast cells. Fig. 7 illustrates the effect of various concentrations of *o*-phenanthroline on hydroxylation. In the case of the pH 8.0 assay without added Fe^{2+}, 2.5 μM *o*-phenanthroline inhibited hydroxylation by 50%. The double-reciprocal plots of velocity against tryptophan concentration without added Fe^{2+} in either presence or absence of *o*-phenanthroline are shown in Fig. 8. Inhibition by *o*-phenanthroline is competitive with respect to tryptophan and K_i was found to be 1.2 μM. Fig. 9 shows that Me_2PteH_4 also counter-

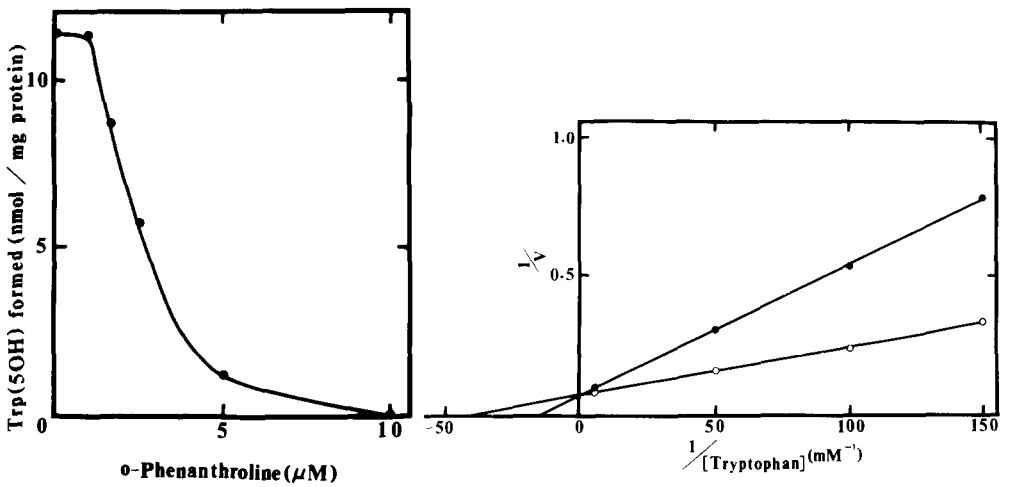

Fig. 7. Inhibition of tryptophan hydroxylase by *o*-phenanthroline. The reaction was carried out using 0.12 mg of enzyme protein and 0.2 μmol of tryptophan and 0.2 μmol Me_2PteH_4 at pH 8.0 without added Fe^{2+}. *o*-Phenanthroline was added after all components of reaction were combined. Other experimental conditions are given in a text.

Fig. 8. Double-reciprocal plots against tryptophan concentration in the presence (•) and absence (○) of *o*-phenanthroline. The experimental conditions are the same as in Fig. 7, except that 0.106 mg of enzyme protein and 2 nmol of *o*-phenanthroline were used.

acts the *o*-phenanthroline inhibition which proved to be competitive with respect to Me_2PteH_4 added at $2 \cdot 10^{-4}$ to $8 \cdot 10^{-4}$ M. From this figure K_i was calculated to be 4.53 μM. As shown in Fig. 10, however, in $1/v$ versus $1/O_2$ plots with and without added *o*-phenanthroline the parallel lines were obtained.

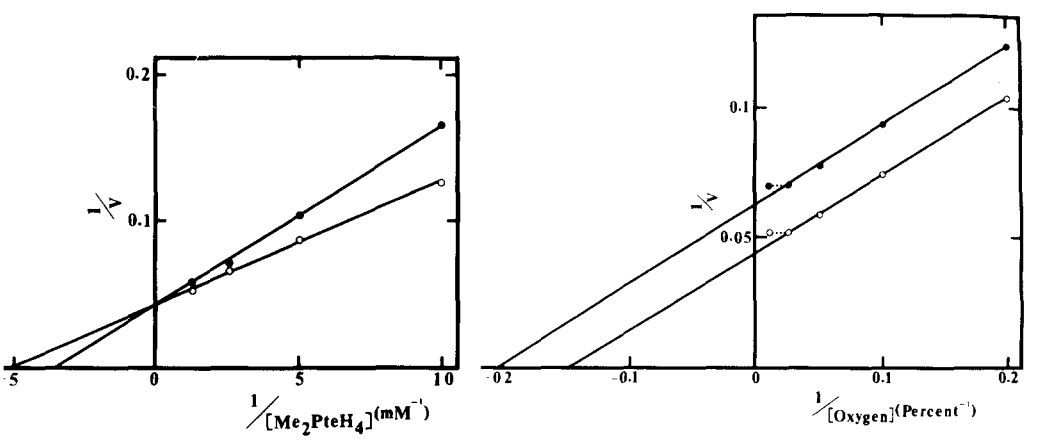

Fig. 9. Double-reciprocal plots against Me_2PteH_4 concentration in the presence (•) and absence (○) of *o*-phenanthroline. The experimental conditions are the same as in Fig. 8.

Fig. 10. Double-reciprocal plots against oxygen concentration in the presence (•) and absence (○) of *o*-phenanthroline. The experimental conditions are the same as in Fig. 8.

Discussion

The first attempt to purify tryptophan hydroxylase from the neoplastic mast cells was made by two groups [3,19]. In 1965, Levine et al. [19] reported in their short communication that tryptophan hydroxylase activity lost completely after breaking cells was largely recovered by addition of Fe^{2+} and Me_2PteH_4. In next year we described the absolute requirement of 2-mercaptoethanol, but not Fe^{2+} for the hydroxylation, together with the finding of the maximum enzyme activity at pH 8.0 [3]. In the subsequent paper by Sato et al. [20], they admitted that 2-mercaptoethanol was essential for tryptophan hydroxylase activity, but insisted again that tryptophan hydroxylation was completely dependent upon exogenous Fe^{2+}. In the present study, the effect of pH in either presence or absence of added Fe^{2+} on the tryptophan hydroxylation was re-examined. The results obtained indicate that pH optimum for full activity was 7.6 to 8.0 in the absence of added Fe^{2+}, depending upon the amounts of 2-mercaptoethanol added, and regardless of the amounts of Tris · HCl or Tris/acetate buffer used, whereas the pH optimum deviated to 6.0 in its presence (Fig. 2). The kinetic data show some differences in K_m values for tryptophan and Me_2PteH_4 except for those for oxygen under the assay conditions with and without added Fe^{2+} (Figs 3, 4, and 5). Another differences in tryptophan hydroxylation with and without added Fe^{2+} are the prominent enzyme activation after anaerobic preincubation in the absence of added Fe^{2+} and the marked inhibition of hydroxylation under oxygen in its presence (Table II and Fig. 5). From these results, it seems likely that Fe^{2+} may not only protect the tryptophan hydroxylation system from H_2O_2-mediated inactivation [12], but also modify the active center of enzyme, probably by altering the affinity of its binding sites toward substrates such as tryptophan and Me_2PteH_4. Thus, the subsequent kinetic studies were carried out under the assay conditions at pH 8.0 without added Fe^{2+}.

Double-reciprocal plots of the initial velocity against either Me_2PteH_4 or oxygen as a variable substrate at several fixed concentrations of tryptophan gave a series of intersecting lines (Figs 4 and 6). In the $1/v$ versus $1/O_2$ plots at changing fixed Me_2PteH_4 concentrations, however, the lines obtained are nearly parallel (Fig. 5). Assuming the ping-pong mechanism for the tryptophan hydroxylation, if either one or two among all three substrates combines with the enzyme reversibly, following irreversible release of a product, the double-reciprocal plots of the initial velocity against the third substrate at different fixed concentrations of either first or second substrate must be parallel. This is not the case, since in the $1/v$ versus either $1/Me_2PteH_4$ or $1/O_2$ at several fixed concentrations of tryptophan or Me_2PteH_4, respectively, the lines obtained intercept (Figs 4 and 6).

Thus, based on the results obtained, the reaction mechanism of tryptophan hydroxylation appears to be of the sequential, rather than ping-pong, type. At present, however, limited kinetic studies have not yet yielded conclusive evidence regarding the order of addition of substrates and the release of products, within the reaction sequences of tryptophan hydroxylation, i.e. whether it is "ordered" or "random".

Another important aspect of the tryptophan hydroxylase mechanism is

related to the marked inhibition of the hydroxylation by *o*-phenanthroline as a metal chelating agent (Fig. 7), in agreement with previous studies on phenylalanine hydroxylase [18] and tyrosine hydroxylase [21,22]. Kinetic studies indicated that the inhibition by this potent inhibitor was competitive with respect to Me_2PteH_4 as well as tryptophan, but appeared to be uncompetitive with respect to molecular oxygen (Figs 8, 9, and 10).

The competition of Me_2PteH_4 with *o*-phenanthroline for tryptophan hydroxylase is in accord with the results of bovine adrenal tyrosine hydroxylase described by Shiman et al. [22], but not with those reported by Taylor et al. [21] who also observed the noncompetitive behavior of tyrosine with *o*-phenanthroline for tyrosine hydroxylase. The results obtained by the latter group can not be compared directly with those in the present experiment, since the assay conditions of each hydroxylase are not same (pH, the amounts of substrate and inhibitor, use of catalase, etc.). In the generic sense, I would interpret simply the present kinetic data as meaning that *o*-phenanthroline may bind at or around the binding sites for Me_2PteH_4 and tryptophan but not for oxygen at the catalytic center of tryptophan hydroxylase. It remains to be elucidated whether or not the binding of *o*-phenanthroline to the above-mentioned active sites is mediated through a chelation mechanism involving the metal ions such as Fe^{2+}, since there has been no current report that the enzyme is bound to any of metal ions. To clarify these aspects, it is necessary to obtain tryptophan hydroxylase in a highly purified state, as has been achieved in rat liver phenylalanine hydroxylase [18]. There is also some uncertainty with respect to the measurements of initial rate of tryptophan hydroxylation, since the non-linearity of the reaction with incubation time was constantly observed under the assay conditions employed (Fig. 1). The alternative short-term assay determining the initial hydroxylation rate is needed for further kinetic evaluations of this enzyme. Finally, it should be pointed out that further studies should include the kinetic analysis of tryptophan hydroxylase using tetrahydrobiopterin which has not been available in the present work, since this naturally occurring cofactor functions during hydroxylation much more actively than Me_2PteH_4 does [12]. Therefore, the present kinetic conclusions regarding the tryptophan hydroxylation mechanism must remain tentative.

Note added in proof

After this article had been submitted for publication, two papers [23,24] have appeared which described certain properties of tryptophan hydroxylases purified partially from *Chromobacterium violaceum* and bovine pineal gland, respectively. It is of interest that the enzyme has also a stringent requirement for sulfhydryl compounds, but not for iron.

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