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FACTORS AFFECTING SUBSTRATE COOPERATIVITY OF RAT LIVER TRYPTOPHAN HYDROXYLASE

P. T. SULLIVAN*, M. V. KESTER AND S. J. NORTON
Department of Chemistry, North Texas State University, Denton, Texas 76203 (U.S.A.)

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SUMMARY

A kinetic study of the partially purified phenylalanine–tryptophan hydroxylase system of rat liver has been undertaken. Under defined experimental conditions the tryptophan response plots exhibit sigmoidal kinetics. The apparent cooperativity is sensitive to temperature and certain "desensitizing" agents. In addition, certain analogs of tryptophan, notably 5-fluorotryptophan and 7-azatryptophan, greatly stimulate the hydroxylation of tryptophan (up to 20-fold) when the concentration of reduced pteridine cofactor is present at moderate concentrations. Phenylalanine and thienylalanine also enhance the hydroxylation of tryptophan at high pteridine levels. These findings are discussed with respect to the proposal that separate binding sites (catalytic and allosteric sites) exist on the quaternary enzyme protein, and that various effectors (including certain tryptophan and phenylalanine analogs), upon preferential combination with the allosteric site(s), exert a modulating influence on the substrate binding and on the consequent catalysis at the catalytic sites.

INTRODUCTION

In 1968 Coulson *et al.*[1] presented evidence which suggested a more complicated model of the phenylalanine–tryptophan hydroxylase system of rat liver than had previously been envisaged by others. Renson, *et al.*[2] had proposed in 1962 that the hydroxylation of tryptophan and phenylalanine by rat liver extracts was mediated by the same protein. Freedland[3] arrived at the same conclusion the following year, even though the two activities were found to have different activators and inhibitors. The model of the phenylalanine–tryptophan hydroxylase system proposed by Coulson *et al.* is that there are separate hydroxylating sites for the benzenoid and indolic sub-

Abbreviation: $AHDMPH_4$, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride.

* Present address: Laboratory of Medicinal Chemistry, College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48104 (U.S.A.).

strates. Further it was proposed that the hydroxylation at each site is allosterically modified by binding at the other site. Recently, Kaufman and Fisher[4] have obtained phenylalanine hydroxylase activity in a form 85–95% pure. They reported that the enzyme exists in two tetrameric isozyme forms, each having molecular weights of approximately 210 000. No mention is made of tryptophan hydroxylase activity in the highly purified preparations. More recently, Barranger *et al.*[5] have shown that three isozyme forms of phenylalanine hydroxylase exist in adult rat liver and that each isozyme form (10-fold purification) exhibits some tryptophan hydroxylase activity.

We wish to report some recent observations in this laboratory regarding the kinetics of hydroxylation of both phenylalanine and tryptophan, and the effects of certain desensitizing agents and amino acid analogs on the reaction kinetics.

MATERIALS

2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine·HCl ($AHDMPH_4$) was obtained from Aldrich Chemical Co., Inc. Guanidine·HCl was obtained from Research Plus and sodium dodecyl sulfate from Sargent Chemical Co. The monoamine oxidase inhibitor, iproniazid phosphate, dithiothreitol, DL-5-fluorotryptophan, DL-7-azatryptophan and β-DL-thienylalanine were obtained from Sigma Chemical Co.

METHODS

Male, random bred Sprague-Dawley rats weighing from 250–350 g were used in this investigation. The rat livers were routinely homogenized in 3 vol. of 0.075 M potassium phosphate, pH 7.4. The supernatant fraction obtained from a 100 000 × *g* centrifugation of the homogenate was used as crude enzyme source. Partially purified preparations of the enzyme were obtained by the following methods: $(NH_4)_2SO_4$ fractionation by the method of Mitoma[6]; $Ca_3(PO_4)_2$ gel-cellulose fractions by the method of Barringer *et al.*[5]. In some instances combinations of these methods were employed to obtain up to a 15-fold purification.

For the hydroxylation of tryptophan the reaction mixtures normally contained: $AHDMPH_4$, 0.8 mM; iproniazid phosphate, 10 mM; dithiothreitol, 2.5 mM; potassium phosphate, pH 7.4, 0.075 M; and L-tryptophan, 2 to 24 mM. The reaction was initiated by the addition of a rate-limiting amount of the partially purified enzyme preparation. The total volume was 0.5 ml, and incubation was for 30 min at 30 °C with shaking in an O_2 atmosphere. The hydroxylation rate was determined to be linear throughout the incubation period. The reaction was terminated by the addition of 0.5 ml of 20% trichloroacetic acid. Hydroxyindole concentration was determined colorimetrically[7]. The reaction mixture for the hydroxylation of phenylalanine was the same as for tryptophan hydroxylation except phenylalanine (0.1–1.0 mM) was substituted for tryptophan. The reaction was again initiated by the addition of the partially purified enzyme preparation and was terminated as above after a 10-min incubation. The tyrosine formed was determined either colorimetrically[8] or fluorometrically[9]. In all assays substrate and enzyme blanks were included.

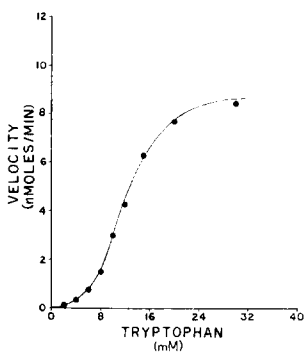
The following methods were also employed: tryptophan pyrrolase assay, the procedure of Schultz and Feigelson[10] which involves the spectrophotometric deter-

mination of N-formylkynurenine and kynurenine; tryptophan transaminase and L-amino acid oxidase assays, the procedure of Lin *et al.*[11] which employs a spectrophotometric determination of indolepyruvic acid; and tryptophan decarboxylase assay, a procedure involving extraction of reaction mixtures into *n*-butanol as described by Udenfriend, *et al.*[7] with subsequent detection of tryptamine by paper chromatography using *n*-butanol–acetic acid–water (4:1:1, v/v/v).

The short column of a Beckman Model 120C amino acid analyzer was used to determine tryptophan. An appropriate dilution of the reaction mixture was applied to the column and citrate buffer, pH 5.25, was used to elute the amino acid.

RESULTS AND DISCUSSION

A typical substrate response plot obtained for tryptophan hydroxylation is shown in Fig. 1. It is noteworthy that the plot is sigmoidal, indicating a possible cooperative effect exerted by the substrate, tryptophan, on its own hydroxylation. The apparent cooperative effect of tryptophan was found to be temperature dependent (Fig. 2), and typical hyperbolic plots are obtained as the incubation temperature is increased from 30 to 37 to 43 °C.



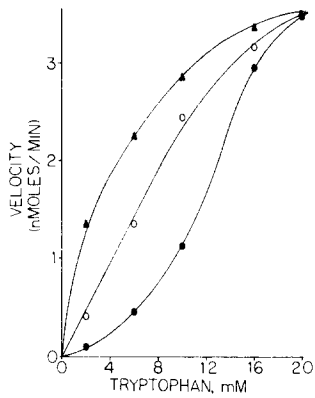
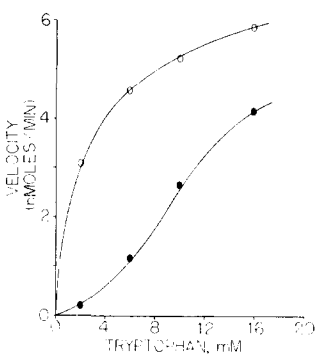


Fig. 1. Typical tryptophan response plot. Reaction velocity is expressed in terms of nmoles of 5-hydroxyindole formed per min. The usual procedures and incubation mixtures were employed as described in Methods.

Fig. 2. Effect of incubation temperature on hydroxylation of tryptophan. Reaction velocity is expressed in terms of nmoles of 5-hydroxyindole formed per min. The usual procedures and incubation mixtures were employed as described in Methods. ●—●, hydroxylation of tryptophan at 30 °C; ○—○, hydroxylation of tryptophan at 37 °C; ▲—▲, hydroxylation of tryptophan at 43 °C.

The effects of several agents known to affect tertiary and quaternary structure (desensitizing agents) were investigated in the hydroxylation reactions for both phenylalanine and tryptophan. The agents employed were sodium dodecyl sulfate, guanidine·HCl and *n*-propanol. The most dramatic effect on the kinetics was exhibited by *n*-propanol (0.5 M in the reaction mixture) for the hydroxylation of tryptophan (Fig. 3). Similar effects were noted for sodium dodecyl sulfate and guanidine·HCl at 0.3% and 0.25 M, respectively, but the magnitude of the enhancements of hydroxylase

activity by the latter agents was somewhat less. In several experiments the rate of tryptophan hydroxylation (at low tryptophan concentrations) was enhanced 20- to 30-fold by *n*-propanol. Other alcohols, including ethanol, *n*-butanol, and various secondary and tertiary alcohols, gave similar, but lower, enhancements. The effect of all desensitizing agents was reversible by dialysis of the treated enzyme preparations prior to assay.



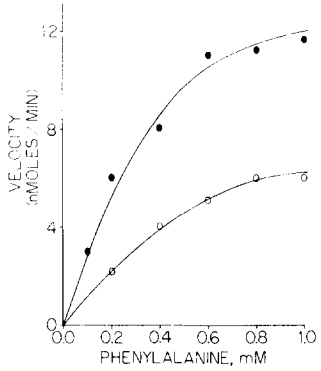


Fig. 3. Effect of *n*-propanol on the hydroxylation of tryptophan. Reaction velocity is expressed in terms of nmoles of 5-hydroxyindole formed per min. The usual procedures and incubation mixtures were employed as described in Methods. ●—●, hydroxylation of tryptophan; ○—○, hydroxylation of tryptophan in the presence of *n*-propanol (0.5 M).

Fig. 4. Effect of *n*-propanol on the hydroxylation of phenylalanine. Reaction velocity is expressed in terms of nmoles of tyrosine formed per min. The usual procedures and incubation mixtures were employed as described in Methods. ○—○, hydroxylation of phenylalanine; ●—●, hydroxylation of phenylalanine in the presence of 0.5 M *n*-propanol.

Of the desensitizing agents tested, only *n*-propanol had any pronounced effect on the hydroxylation of phenylalanine (Fig. 4). The enhancement is definite, but of significantly lower magnitude than that given for tryptophan hydroxylation. In the case of phenylalanine, the hydroxylation kinetics (either with or without added *n*-propanol) are typical Michaelis–Menten, hyperbolic kinetics.

The possibility that the sigmoidal response is due to competition by other enzymes utilizing tryptophan or its hydroxylation products is unlikely. For example, as seen in Table I the loss of the substrate, tryptophan, by cleavage, transamination, oxidative deamination and decarboxylation has been found to be insignificant. Only tryptophan pyrrolase was detectable under our usual reaction conditions employing a partially purified enzyme preparation, but the activity was not inhibited by *n*-propanol nor was it sufficiently active to remove significant amounts of substrate from the reaction mixture. Monoamine oxidase is routinely inhibited by the inclusion of iproniazid phosphate in the reaction mixture. The concentrations of the indoles and hydroxyindoles remain constant, even after incubation for 1 h in the reaction mixture, with or without *n*-propanol. Perhaps the most revealing studies were carried out with the amino acid analyzer. At every tryptophan concentration studied in the reaction mixtures, there was almost total recovery of this amino acid after incubation with or without *n*-propanol; the amount of tryptophan remaining was consistent with the amount of product formed as determined colorimetrically.

TABLE I

STUDIES OF POSSIBLE PROCESSES AFFECTING KINETICS OF TRYPTOPHAN HYDROXYLATION

Studies conducted	*Results*
Enzyme studies	
Tryptophan pyrrolase activity[a]	(a) No propanol inhibition (b) No effect on tryptophan hydroxylation kinetics when pyrrolase activity induced 3 to 10-fold[b]
Tryptophan transaminase-oxidase activity[c]	No detectable activity
Tryptophan decarboxylase activity (paper chromatography[d]	No detectable activity
Analytical studies	
Colorimetric determination of indole survival of incubation period[e]	95–100% of indole added is present after incubation (No propanol effect)
Colorimetric determination of hydroxyindole survival of incubation period[d]	No loss of hydroxyindoles (No propanol effect)
Amino acid analyzer determination of tryptophan survival of incubation period	>95% of tryptophan recovered (No propanol effect)

[a] See ref. 10.
[b] See ref. 12.
[c] See ref. 11.
[d] See ref. 7.
[e] See ref. 7 and 13.

Further, it was found that *n*-propanol, added to reaction mixtures after termination of the enzymatic process with trichloroacetic acid, had no effect on the assay of hydroxyindoles. No hydroxyindoles could be extracted from the precipitated protein. An effect on hydroxylation kinetics was observed only when this alcohol was present during the enzyme reaction.

It is well known that nonlinear kinetic behavior can arise from two-substrate reactions when a ternary complex is formed in a random order process[14]. The substrate-velocity plot may in some cases be sigmoidal. Since the phenylalanine–tryptophan hydroxylase system is complicated by the requirement of three substrates (tryptophan, reduced pteridine, and O_2) for reaction, a complete kinetic analysis is obviously difficult. In view of the pronounced effects of guanidine·HCl, sodium dodecyl sulfate, heat treatment, and alcohols on the hydroxylation kinetics of tryptophan hydroxylase, and in view of the fact that these agents give rise to linear double-reciprocal plots, it is reasonable to assume that their effect on the system is not simply to establish an obligatory pathway in the reaction process. Rather, it is believed that these desensitizing agents or treatments modify or negate enzyme-substrate cooperativity such that typical Michaelis–Menten kinetics result.

The effects of various natural amino acids and amino acid analogs were studied in the tryptophan hydroxylase system. Neither tyrosine nor any of the non-aromatic amino acids studied, had any measurable effect on the hydroxylation rate of tryptophan. However, phenylalanine, which inhibits tryptophan hydroxylation when the reduced pteridine cofactor is at low concentrations (0.7 mM), substantially enhances the rate of hydroxylation of tryptophan when the concentration of reduced pteridine is increased to 3.5 mM. As seen in Fig. 5A the response plot approaches a hyperbolic

shape when phenylalanine (0.4 mM) is added to the reaction mixture. In other studies it was found that thienylalanine, at the same concentration as phenylalanine, gives an even greater enhancement of hydroxylation at low tryptophan levels. The enhancement of tryptophan hydroxylation by both phenylalanine and thienylalanine disappears at higher incubation temperatures (*e.g.* 43 °C), and in the presence of 0.5 M *n*-propanol (Fig. 5B).

Fig. 5. Effect of L-phenylalanine on the hydroxylation of tryptophan with low and high levels of $AHDMPH_4$ and with and without *n*-propanol. Reaction velocity is expressed in terms of nmoles of 5-hydroxyindole formed per min. The usual procedures and incubation mixtures were employed as described in the Methods section. A, Without *n*-propanol. ●—●, 0.7 mM $AHDMPH_4$; ○—○, 0.7 mM $AHDMPH_4$ with 0.4 mM L-phenylalanine; ▲—▲, 3.5 mM $AHDMPH_4$; △—△, 3.5 mM $AHDMPH_4$ with 0.4 mM L-phenylalanine. B, With 0.5 M *n*-propanol. ●—●, 0.7 mM $AHDMPH_4$; ○—○, 0.7 mM $AHDMPH_4$ with 0.4 mM L-phenylalanine; ▲—▲, 3.5 mM $AHDMPH_4$; △—△, 3.5 mM $AHDMPH_4$ with 0.4 mM L-phenylalanine.

Fig. 6. Effect of 7-azatryptophan and 5-fluorotryptophan on the hydroxylation of tryptophan. Reaction velocity is expressed in terms of nmoles of 5-hydroxyindole formed per min. The usual procedures and incubation mixtures were employed as described in the Methods section. Controls were carried out which indicated no interference with the colorimetric determination of 5-hydroxyindoles by either analog. ●—●, hydroxylation of tryptophan; ○—○, hydroxylation of tryptophan in the presence of 8 mM DL-7-azatryptophan; ▲—▲, hydroxylation of tryptophan in the presence of 8 mM DL-5-fluorotryptophan.

The studies given above with phenylalanine and thienylalanine support the proposal that these amino acids bind with an allosteric site[1], effecting an enhancement of the catalytic rate of tryptophan hydroxylation. Since tryptophan has been shown to exert a cooperative effect on its own hydroxylation, a natural conclusion is that this positive effect is also mediated through tryptophan binding at a secondary or allosteric site. In order to ascertain if an allosteric binding occurs with the indole ring amino acid, two tryptophan analogs, 7-azatryptophan and 5-fluorotryptophan, were studied with respect to their effects on tryptophan hydroxylation. Neither analog is hydroxylated to any significant extent by rat liver tryptophan hydroxylase. One might anticipate these analogs to exert an inhibitory effect on tryptophan hydroxylation because of their obvious structural similarity to the natural amino acid. Not only did we find no inhibition of tryptophan hydroxylase activity, but rather, a substantial enhancement of the hydroxylation of tryptophan was found, particularly with 5-fluorotryptophan (Fig. 6). Controls in each study indicated that there was no inter-

ference of the assay method by either of the tryptophan analogs. In the case of 5-fluorotryptophan, the change from sigmoidal to hyperbolic kinetics through the influence of this analog is very similar to the effect mediated by *n*-propanol. The extent of enhancement at low tryptophan concentrations is also on the order 20- to 30-fold

The action of 5-fluorotryptophan on the kinetics of tryptophan hydroxylase is similar to that of α-methyltryptophan on the activity of tryptophan pyrrolase[15]. The usual sigmoidal tryptophan response plots for the latter enzyme are changed to hyperbolic plots in the presence of α-methyltryptophan. This change has been proposed to be the result of allosteric binding of this analog with the enzyme protein, giving rise to an increased affinity of the active site for the substrate, O_2.

It has also been found, and is believed significant, that the enhancement of tryptophan hydroxylation by either 5-fluorotryptophan or *n*-propanol is not additive. This finding suggests that the net effect of either *n*-propanol or 5-fluorotryptophan is the same or at least very similar; namely, that desensitization by *n*-propanol causes a conformational change in the tertiary-quaternary system conducive to an enhancement of tryptophan hydroxylase activity; similarly, binding of tryptophan analogs (or phenylalanine and certain of its analogs) with an allosteric site causes a conformational change in protein structure, again resulting in enhancement of tryptophan hydroxylase activity. The "allosteric site" explanation is consistent with the cooperative effect that tryptophan exerts on its own hydroxylation. It appears logical to propose that the affinity of the allosteric site for the fluoro-analog of tryptophan is much greater than that for the catalytic site, since there is no apparent competition for the hydroxylation site.

Tourian[16] has given evidence that phenylalanine combines, in a time-dependent process, with an allosteric site on the enzyme and thus exerts a positive influence on the hydroxylation of phenylalanine. This effect, which is not pronounced in our system, is in accord, nevertheless with our observation of the enhancement by *n*-propanol. Thus, activation of the active site by conformational changes exerted by the alcohol would be exhibited by an enhanced rate of hydroxylation of the substrate, phenylalanine. From the observations reported here, it now appears that a similar and even more pronounced allosteric effect is involved in the hydroxylation of tryptophan. Questions as to whether the catalytic and allosteric sites on the enzyme protein are common to both benzenoid and indole ring systems, or are separate sites, can be debated. The biological significance of the allosteric activation of rat liver tryptophan hydroxylase *in vitro* remains to be determined.

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