MOLECULAR PHARMACOLOGY, 14, 99-110

Activation of Tryptophan Hydroxylase by Adenosine Triphosphate, Magnesium, and Calcium

MICHEL HAMON, SYLVIE BOURGOIN, F. HÉRY, AND G. SIMONNET

Groupe NB, Institut National de la Santé et de la Recherche Médicale U114, Collège de France, 75231 Paris, France

(Received May 17, 1977)
(Accepted August 3, 1977)

SUMMARY

HAMON, MICHEL, BOURGOIN, SYLVIE, HÉRY, F. & SIMONNET, G. (1978) Activation of tryptophan hydroxylase by adenosine triphosphate, magnesium and calcium. *Mol. Pharmacol.*, 14, 99-110.

The addition of 0.5 mM ATP and 5 mM Mg^{2+} to a crude extract (35,000 × *g* supernatant) of a semipurified preparation (obtained by ammonium sulfate fractionation and Sephadex G-200 gel filtration) of tryptophan hydroxylase from rat brain stem induced a [illegible] increase in this enzymatic activity. The tryptophan hydroxylase remained in [illegible]vated state upon the removal of ATP and Mg^{2+} by filtration through a Sephadex [illegible]olumn. The activation probably involved a phosphorylation reaction, since [illegible]denosine, a well-known protein kinase inhibitor, reduced the effects of ATP and Mg^{2+}. In addition, when ATP was replaced by 5'-adenylylimidodiphosphate in the activating mixture, the activity of tryptophan hydroxylase remained unchanged, indicating that the terminal phosphate of ATP was required in the activating process. Although exogenous cyclic 3',5'-AMP-dependent protein kinase was able to increase further the activating effect of ATP and Mg^{2+} under some conditions (notably on the semipurified tryptophan hydroxylase), cyclic AMP failed to exert any effect on the enzyme in a crude extract. By contrast, when Ca^{2+} was included in the assay mixture, the stimulatory effect of ATP and Mg^{2+} on tryptophan hydroxylase activity was enhanced whatever the enzyme source. This effect of Ca^{2+} was maximal (25–30%) with a 10 μM concentration of the cation. These results strongly suggest that a Ca^{2+}-dependent protein kinase may be involved in the activation of tryptophan hydroxylase induced by phosphorylating conditions. Kinetic analyses of tryptophan hydroxylase revealed that the apparent affinities of this enzyme for both tryptophan and DL-6-methyl-5,6,7,8-tetrahydropterin were increased under phosphorylating conditions.

INTRODUCTION

Tryptophan hydroxylase (L-tryptophan 5-monooxygenase, EC 1.14. 16.4) catalyzes the rate-limiting step in the biosynthesis of serotonin in the central nervous system. The key role of this enzyme in the regulation of serotonin synthesis has been stressed many times in the literature. In particular, several authors (1-3) have shown that the accelerated synthesis of serotonin occurring in the forebrain during electrical stimulation of the raphe nuclei resulted from an increase in the rate of tryptophan hydroxylation. Conversely, a decrease in this enzymatic reaction was observed *in vivo* in terminal areas when

This research was supported by grants from the Institut National de la Santé et de la Recherche Médicale, DGRST, DRME, and the Société des Usines Chimiques Rhône-Poulenc.

0026-895X/78/0141-0099$02.00/0

the firing within serotoninergic neurons had been reduced or suppressed by pharmacological or surgical manipulations (4–6). All these data strongly suggest that the activity of tryptophan hydroxylase is dependent on the rate of nerve impulse flow in serotoninergic neurons.

Since the influx of Ca^{2+} into the terminals is directly related to the frequency of action potentials in neurons, this cation may be involved in the control of tryptophan hydroxylase activity in serotoninergic neurons. In fact, Ca^{2+} was shown to increase the activity of tryptophan hydroxylase *in vitro* (7-9) and, according to Knapp *et al.* (8), it might be responsible for the rapid changes in tryptophan hydroxylase activity seen *in vivo* during modifications of the nerve impulse flow in serotoninergic neurons. However, since the activating effect of Ca^{2+} observed in the addition experiments *in vitro* resulted from irreversible partial proteolysis of tryptophan hydroxylase by a Ca^{2+}-dependent neutral proteinase, the hypothesis of Knapp *et al.* (8) has recently been challenged (9).

Extensive studies on the regulation of tyrosine hydroxylase activity in dopaminergic and noradrenergic neurons led to the proposal that cyclic 3′,5′-AMP-dependent phosphorylation of some protein, and possibly of tyrosine hydroxylase itself, is involved in this process. Indeed, the electrical stimulation of both peripheral noradrenergic neurons (10, 11) and central noradrenergic (12) and dopaminergic (13) neurons induced an activation of tyrosine hydroxylase which closely resembled that occurring *in vitro* in the presence of ATP, Mg^{2+}, and cyclic AMP. When soluble tyrosine hydroxylase from unstimulated tissues was incubated with ATP, Mg^{2+}, and cyclic AMP, its apparent affinity for the pterin cofactor increased significantly (14–16), up to the degree found for enzyme extracted from electrically stimulated preparations (10–13).

These findings led to the search for similar effects of phosphorylating conditions on tryptophan hydroxylase. As already reported for tyrosine hydroxylase (14, 16), we found that the addition of ATP and Mg^{2+} to the assay mixture activated the soluble tryptophan hydroxylase from the rat brain stem. However, cyclic AMP was not required in this process. In fact, the stimulatory effect of ATP and Mg^{2+} on tryptophan hydroxylase activity was enhanced by the addition of low concentrations (5–50 μM) of Ca^{2+}, suggesting that a Ca^{2+}-dependent protein kinase is involved in the activating process.

METHODS

ATP and 5′-adenylylimidodiphosphate were obtained from Boehringer. Theophylline, catalase (14,400 units/mg), cyclic 3′,5′-AMP, 8-bromo-cyclic AMP, N^6,2′-*O*-dibutyryl cyclic AMP, cyclic 3′,5′-GMP, N^6,2′-*O*-dibutyryl cyclic GMP, cyclic AMP-dependent protein kinase (from beef heart), EGTA[1], α-casein, and 5,5-diphenylhydantoin were purchased from Sigma.

Other compounds were adenosine (Schwarz/Mann), *N*′-methyl-*N*-(3-hydroxybenzyl)hydrazinium dihydrogen phosphate (NSD 1034, Smith and Nephew Research), 8-chlorophenylthio-cyclic [redacted] (ICN), and DL-6-methyl-5,6,7,8-tetrahydropterin (Calbiochem). Sephadex G-25 and G-200 were purchased from Pharmacia. Salts (ammonium sulfate, sodium fluoride, magnesium chloride and acetate, and calcium chloride and acetate) were of the highest purity commercially available (Merck, Prolabo).

Soluble tryptophan hydroxylase was extracted from the brain stems of male Sprague-Dawley rats (250–300 g) by homogenizing tissues in 3 volumes (v/w) of 0.05 M Tris-acetate, pH 7.6, containing 2 mM 2-mercaptoethanol. Homogenization was performed at 4°, using a Polytron PT-10 OD apparatus, for 25 sec at maximal speed. The homogenate was then centrifuged for 30 min at 35,000 × *g* in a Sorvall RC-2B instrument, and the clear supernatant was generally used as the final enzyme source. On occasion the supernatant (1–1.5 ml) was filtered through a Sephadex

[1] The abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; 6-MPH$_4$, DL-6-methyl-5,6,7,8-tetrahydropterin; 5-HTP, 5-hydroxytryptophan; App(NH)p, 5′-adenylylimidodiphosphate.

G-25 column (0.9 × 14 cm) in order to eliminate small contaminating molecules in the tryptophan hydroxylase solution. The same buffer, i.e., 0.05 M Tris-acetate, pH 7.6, containing 2 mM 2-mercaptoethanol, was used for equilibration of the Sephadex gel and migration of proteins. The colored eluate (corresponding to the void volume of the gel) was used as the tryptophan hydroxylase source. Preliminary experiments with α-[^{32}P]ATP (10 Ci/mmole, New England Nuclear) showed that this filtration procedure effectively eliminated contaminating small molecules from the original protein solution without significantly changing its tryptophan hydroxylase activity (expressed per milligram of protein).

Tryptophan hydroxylase was partially purified by ammonium sulfate fractionation and Sephadex G-200 gel filtration. The entire procedure was performed at 4°. A saturated ammonium sulfate solution (pH 7.2) was progressively added to the 30,000 × g supernatant of a rat brain stem homogenate until 20% saturation was achieved. After stirring for 30 min, the mixture was centrifuged (30,000 × g, 20 min), and the resulting supernatant was brought to 50% saturation with the saturated ammonium sulfate solution. After stirring and centrifugation as before, the pellet was finally dissolved in a small volume (2–3 ml) of 0.05 M Tris-acetate, pH 7.6, containing 2 mM 2-mercaptoethanol and passed through a column of Sephadex G-200 (2.6 × 60 cm). Proteins were eluted with the Tris-acetate buffer (0.05 M, pH 7.6, containing 2 mM 2-mercaptoethanol) used for equilibration of the gel. Fractions (corresponding to 10–20 ml) containing the highest enzyme activity were pooled and used as the semipurified tryptophan hydroxylase preparation.

Tryptophan hydroxylase was assayed according to Gál and Patterson (17) with slight modifications (9). Routine assays were performed with 0.15 mM tryptophan and 0.16 mM 6-MPH$_4$. The final product, 5-hydroxytryptophan, was measured using a spectrofluorometric method (18). The formation of 5-HTP was proportional to time (for at least the first 15 min of incubation at 37°) and protein concentration (0.3–1.2 mg/ml in the assay mixture) under all experimental conditions used in the present study.

Experiments were repeated at least twice, with triplicate determinations for each condition. The range of variations among triplicate values never exceeded 3%.

Enzymatic activity is expressed as nanomoles of 5-HTP formed per milligram of protein in the assay mixture per 15 min of incubation at 37°. For determination of the kinetic parameters (apparent K_m, V_{max}) of tryptophan hydroxylase for tryptophan, the concentration of 6-MPH$_4$ was raised to 0.32 mM and the tryptophan concentration was varied between 20 μM and 0.5 mM. Conversely, the concentration of tryptophan was maintained at 0.5 mM and that of 6-MPH$_4$ was varied between 50 μM and 0.32 mM for determination of the kinetic parameters of the enzyme for its pterin cofactor. In both cases parameters were calculated by linear regression analysis of double-reciprocal (Lineweaver-Burk) plots.

The possible occurrence of proteolysis under the various conditions used for the tryptophan hydroxylase assay and activation was checked as previously described (9); the release of free tyrosine in the incubation mixture was used as an index of proteolysis. Tyrosine was measured by a spectrofluorometric method (19).

Proteins were determined (20) with bovine serum albumin as the standard.

Statistical calculations (Student's t-test) were done as described by Snedecor and Cochran (21).

RESULTS

Effects of ATP and Mg^{2+} on tryptophan hydroxylase activity. The addition of ATP (10 μM–1 mM) to the tryptophan hydroxylase assay mixture resulted in discrete changes in enzymatic activity. Slight inhibition (8–12%) was noted in the presence of 0.5–1 mM ATP. By contrast, tryptophan hydroxylase activity increased slightly when Mg^{2+} (chloride or acetate) was included in the assay mixture. The maximal increase (10–20%) occurred with 5 mM

Mg^{2+} (Table 1). The changes induced by ATP or Mg^{2+} were observed not only in the crude enzyme preparation (35,000 × *g* supernatant) but also in the semipurified tryptophan hydroxylase (Table 1). When ATP and Mg^{2+} were added together, the enzymatic activity increased by 40–50%. The maximal stimulation of tryptophan hydroxylase activity occurred in the presence of 5 mM Mg^{2+} and 0.5 mM ATP. As shown in Table 1, this effect was observed in the 35,000 × *g* supernatant, the Sephadex G-25 filtrate, and the semipurified preparation.

Previous incubation of the tryptophan hydroxylase crude 35,000 × *g* supernatant with 0.5 mM ATP and 5 mM Mg^{2+} at 30° for 7 min resulted in a persistent increase in its activity (Table 2). Indeed, filtration of the previously incubated mixture through a Sephadex G-25 column in order to remove small molecules (notably ATP and Mg^{2+}) did not abolish the activation induced by ATP and Mg^{2+} during the prior incubation period (Table 2). Under these conditions the addition of sodium fluoride [a well-known inhibitor of protein phosphatase activity (22)] to the enzyme solution throughout the experiment (prior incubation plus enzyme assay) slightly enhanced the activation of tryptophan hydroxylase by ATP and Mg^{2+} (Table 2).

Effect of pH on activation of tryptophan hydroxylase by ATP and Mg^{2+}. In assays below pH 7.3, the addition of ATP and Mg^{2+} exerted almost no effect on tryptophan hydroxylase activity. The ATP- and Mg^{2+}-induced activation was obvious at pH 7.6 (40%) and then progressively increased as a function of pH (80% stimulation at pH 8.3) (Fig. 1). Consequently the optimal pH for tryptophan hydroxylase activity in the 35,000 × *g* supernatant was shifted from 7.6 to 7.9 when ATP and Mg^{2+} were included in the assay mixture (Fig. 1). In another set of experiments, the 35,000 × *g* supernatant of a rat brain stem homogenate was first incubated at pH 7.6 with 0.5 mM ATP and 5 mM Mg^{2+} and then filtered through a Sephadex G-25 column as described above. Determination of the optimal pH for the resulting activated tryptophan hydroxylase indicated that this value was returning to 7.6. Therefore the shift in optimal pH was observed only when ATP and Mg^{2+} were included in the assay mixture, since the gel filtration resulted in the removal of

TABLE 1

Effects of ATP and Mg^{2+} on tryptophan hydroxylase activity

The enzyme was prepared to various stages of purification as described in METHODS. ATP (0.5 mM) and/or magnesium acetate (5 mM) were added to the assay mixture without previous incubation. In all cases the pH of the assay mixture was 7.6. Tryptophan hydroxylase activity was assayed in the presence of 0.15 mM tryptophan and 0.16 mM 6-MPH_4.

Additions	35,000 × *g* supernatant	Sephadex G-25 filtrate	Semipurified enzyme
	nmoles 5-HTP/mg protein/15 min (% control)		
None	2.17 (100)	2.58 (100)	6.08 (100)
ATP	2.04 (94)	2.37 (92)	5.59 (92)
Mg^{2+}	2.40 (111)	2.89 (112)	7.31 (120)
ATP, Mg^{2+}	3.36 (155)	3.62 (140)	9.48 (156)

TABLE 2

Activation of tryptophan hydroxylase by prior incubation with ATP, Mg^{2+}, and NaF

The 35,000 × *g* supernatant of a rat brain stem homogenate was first incubated at 30° for 7 min in the absence and presence of ATP (0.5 mM), magnesium chloride (5 mM), and/or sodium fluoride (12.5 mM). It was then filtered through a Sephadex G-25 column (0.9 × 14 cm), and the colored eluate was used as the source of tryptophan hydroxylase. The enzyme was assayed at pH 7.6 with 0.15 mM tryptophan and 0.16 mM 6-MPH_4 in the absence and presence of ATP, magnesium acetate, and sodium fluoride at the concentrations used for the previous incubation. Each value is the mean of triplicate determinations.

Additions to medium before Sephadex G-25 filtration	Additions to assay mixture	Tryptophan hydroxylase activity
		nmoles 5-HTP/mg protein/15 min (% control)
None	None	1.90 (100)
ATP, Mg^{2+}	None	2.52 (133)
ATP, Mg^{2+}, NaF	NaF	2.69 (142)
None	ATP, Mg^{2+}	2.81 (148)
None	ATP, Mg^{2+}, NaF	2.75 (145)

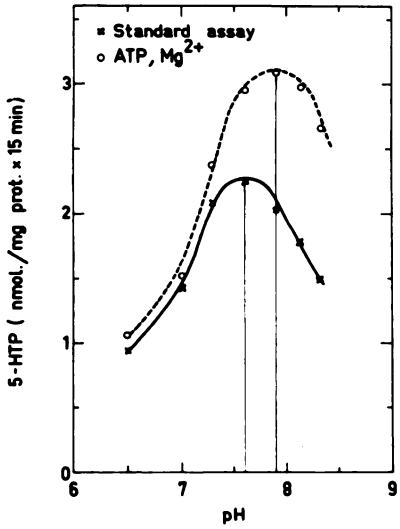

FIG. 1. *Effects of pH on tryptophan hydroxylase activity in the absence* (×) *and presence* (○) *of ATP (0.5 mM) and Mg^{2+} (5 mM)*

ATP and magnesium acetate were added to the assay mixture with no previous incubation. Tryptophan hydroxylase in the 35,000 × *g* supernatant was assayed at various pH values with 0.15 mM tryptophan and 0.16 mM 6-MPH_4. Each point is the mean of triplicate determinations.

these compounds from the enzyme solution.

In some of the following experiments tryptophan hydroxylase activity was measured at pH 8.1, since the activating effects of ATP and Mg^{2+} were higher than at pH 7.6 when both compounds were added to the assay mixture.

Involvement of a protein kinase in activation of tryptophan hydroxylase induced by ATP and Mg^{2+}. The addition of cyclic AMP (1 μM–0.2 mM) with or without exogenous cyclic AMP-dependent protein kinase (10–40 μg/mg of protein in the assay mixture) did not change the stimulatory effect of 5 mM Mg^{2+} and 0.5 mM ATP on the activity of tryptophan hydroxylase in the 35,000 × *g* supernatant. However, when the concentration of ATP was lower than 0.5 mM, the addition of cyclic AMP and the exogenous protein kinase (20 μg) potentiated the effect of ATP and Mg^{2+} on tryptophan hydroxylase activity (Fig. 2). In the case of the semipurified tryptophan hydroxylase, the combined addition of cyclic AMP and exogenous protein kinase also increased the activation induced by ATP and Mg^{2+} (Fig. 3). However, in contrast to observations with the crude extract, the effect of the exogenous protein kinase on tryptophan hydroxylase eluted from the Sephadex G-200 column was still present when optimal concentrations of ATP (0.5 mM) and Mg^{2+} (5 mM) were included in the assay mixture (Fig. 3).

In spite of the failure of exogenous protein kinase to potentiate the stimulatory effect of 0.5 mM ATP and 5 mM Mg^{2+} on tryptophan hydroxylase activity in the crude 35,000 × *g* supernatant, evidence for the involvement of an endogenous protein kinase in these effects was obtained in experiments with adenosine and App(NH)p. Adenosine, a well-known inhibitor of protein kinases (22), markedly inhibited the stimulatory effect of Mg^{2+} and ATP on tryptophan hydroxylase activ-

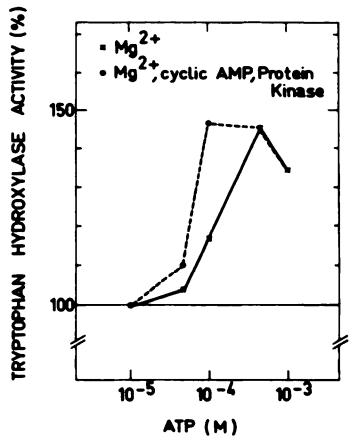

FIG. 2. *Stimulatory effect of exogenous cyclic AMP-dependent protein kinase on tryptophan hydroxylase activity in the presence of low concentrations of ATP*

Tryptophan hydroxylase (in the 35,000 × *g* supernatant) was assayed at pH 7.6 with 0.15 mM tryptophan, 0.16 mM 6-MPH_4, and 5 mM magnesium chloride in the presence of various concentrations of ATP. For each concentration of ATP, the assay was performed in the absence and presence of cyclic AMP (0.5 mM) and cyclic AMP-dependent protein kinase (20 μg/mg of protein in the assay mixture). Tryptophan hydroxylase activity is expressed as a percentage of corresponding values in the absence of ATP. Each point is the mean of triplicate determinations.

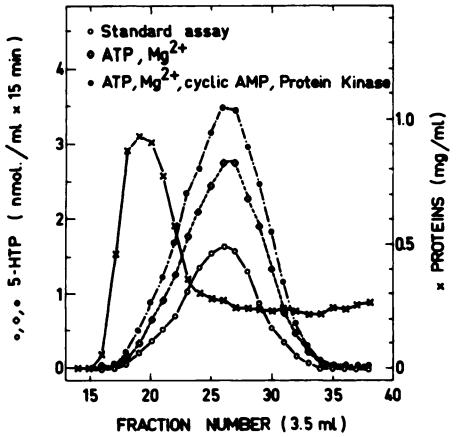

FIG. 3. *Effects of ATP, Mg^{2+}, cyclic AMP, and exogenous cyclic AMP-dependent protein kinase on tryptophan hydroxylase eluted from Sephadex G-200*

The protein of the 35,000 × *g* supernatant (9 ml) precipitated between 20% and 50% saturation with ammonium sulfate was filtered through a Sephadex G-200 column (see METHODS). Tryptophan hydroxylase was assayed in each fraction at pH 7.6 with 0.2 mM tryptophan and 0.16 mM 6-MPH_4 in the absence and presence of ATP (0.5 mM) and magnesium acetate (5 mM), with or without cyclic AMP (0.2 mM) and exogenous cyclic AMP-dependent protein kinase (20 μg). The enzymatic activity is expressed as nanomoles of 5-HTP formed per 15 min by the amount of enzyme contained in 1 ml of each fraction.

ity (Table 3). In addition, replacing ATP with an equimolar concentration of App(NH)p resulted in a decrease in tryptophan hydroxylase activity, to the level seen in the presence of 5 mM Mg^{2+} alone (Table 3).

Effects of cyclic nucleotides on tryptophan hydroxylase activity in the presence and absence of ATP and Mg^{2+}. Neither cyclic AMP, dibutyryl cyclic AMP, 8-bromo-cyclic AMP, nor 8-chlorophenylthio-cyclic AMP exerted any effect on tryptophan hydroxylase activity when added to the assay mixture with or without ATP and Mg^{2+}. These experiments were performed with crude and semipurified preparations of tryptophan hydroxylase, using a wide range of concentrations of the cyclic adenosine nucleotides (1 μM–0.2 mM).

Similarly, neither cyclic GMP (1 μM–0.2 mM) nor its dibutyryl derivative (1 μM–0.2 mM) altered the activity of tryptophan hydroxylase in the 35,000 × *g* supernatant of a rat brain stem homogenate, whether the assay was performed in the presence or absence of 0.5 mM ATP and 5 mM Mg^{2+}. Similar results were obtained when theophylline (0.5 mM) was added with any cyclic nucleotide to the enzyme assay mixture.

Effects of Ca^{2+} on tryptophan hydroxylase activity in the presence and absence of ATP and Mg^{2+}. In agreement with previous findings (9), the addition of Ca^{2+} to the tryptophan hydroxylase assay mixture resulted in a significant increase in enzyme activity, provided that the concentration of the cation exceeded 0.2 mM (Fig. 4). In particular, the addition of 1 mM Ca^{2+} to the assay mixture, using the 35,000 × *g* supernatant as the enzyme source, resulted in a 40–60% increase in tryptophan hydroxylase activity (Fig. 4 and Table 4). The release of free tyrosine that occurred in the assay mixture when 1 mM Ca^{2+} and 0.5 mg of α-casein (protease substrate) were included (Table 4) has previously been shown to depend on proteolysis (9). As shown in Table 4, proteolysis did not occur when the Ca^{2+} concentration was reduced to 10 μM, even when ATP and Mg^{2+} were present in the assay mixture.

TABLE 3

Effects of adenosine and App(NH)p on tryptophan hydroxylase activity under activating conditions with ATP and Mg^{2+}

Each compound was added directly to the assay mixture with no prior incubation. Tryptophan hydroxylase activity in the 35,000 × *g* supernatant was measured at pH 7.6 with 0.2 mM tryptophan, 0.16 mM 6-MPH_4, and, as indicated, 5 mM magnesium acetate, 0.5 mM ATP, 1 mM adenosine, or 0.5 mM App(NH)p. Each value is the mean of triplicate determinations.

Additions	Tryptophan hydroxylase activity
	nmoles 5-HTP/mg protein/15 min (% control)
None	2.70 (100)
Mg^{2+}	2.92 (108)
ATP	2.54 (94)
ATP, Mg^{2+}	3.95 (146)
Adenosine	2.43 (90)
Adenosine, ATP, Mg^{2+}	2.92 (108)
App(NH)p	2.54 (94)
App(NH)p, Mg^{2+}	2.93 (109)

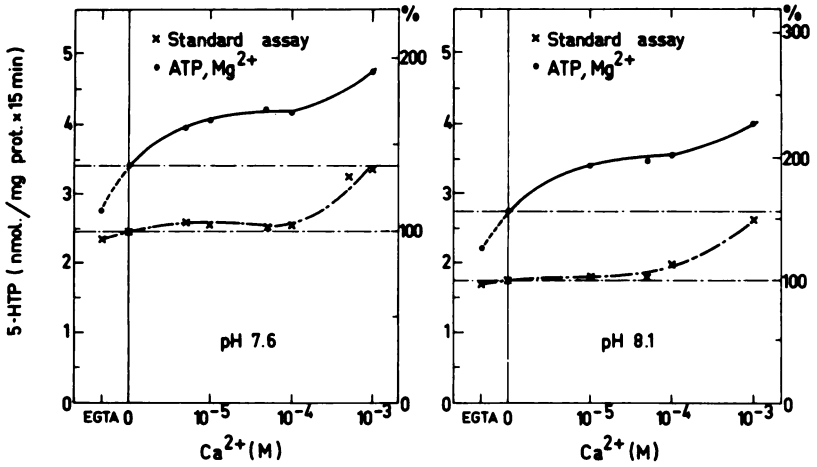

FIG. 4. *Effects of Ca^{2+} on tryptophan hydroxylase activity in the absence and presence of ATP and Mg^{2+} at pH 7.6 or 8.1*

Tryptophan hydroxylase (in the 35,000 × *g* supernatant) was assayed with 0.2 mM tryptophan and 0.15 mM 6-MPH_4. Each compound (0.5 mM ATP, 5 mM magnesium acetate, 50 μM EGTA, 0–1 mM calcium chloride) was added to the assay mixture without prior incubation. Each point is the mean of triplicate determinations.

TABLE 4

Effects of Ca^{2+}, Mg^{2+}, and ATP on release of free tyrosine and formation of 5-HTP in tryptophan hydroxylase assay

Calcium chloride (10 μM or 1 mM), ATP (0.5 mM), and magnesium acetate (5 mM) were added directly to the assay mixture with no prior incubation. The enzyme source was the 35,000 × *g* supernatant of a rat brain stem homogenate. The assay mixture for the release of free tyrosine was the same as for the measurement of tryptophan hydroxylase activity, except that L-tryptophan was omitted and α-casein (0.5 mg) was included. The incubation proceeded for 30 min at 37°. Free tyrosine was then estimated as previously described (9). Each value is the mean of triplicate determinations. In the tryptophan hydroxylase assays the concentrations of tryptophan and 6-MPH_4 were 0.2 mM and 0.16 mM, respectively. Standard assay conditions (pH 7.6) were used. Each value is the mean of triplicate determinations.

Additions	Tyrosine	Tryptophan hydroxylase activity
	nmoles/mg protein (% control)	*nmoles 5-HTP/mg protein/15 min (% control)*
None	2.34 (100)	2.70 (100)
Ca^{2+} (10 μM)	2.18 (93)	2.73 (101)
ATP, Mg^{2+}	2.18 (93)	3.95 (146)
ATP, Mg^{2+}, Ca^{2+} (10 μM)	2.33 (99)	4.55 (169)
Ca^{2+} (1 mM)	4.26 (182)	4.24 (157)

In the presence of ATP and Mg^{2+}, Ca^{2+} was still found to increase tryptophan hydroxylase activity. However in contrast to the effect observed in the presence of Ca^{2+} alone, only a 5–10 μM concentration of Ca^{2+} was required to induce significant activation of tryptophan hydroxylase when ATP and Mg^{2+} were included in the assay mixture (Fig. 4). As shown in Fig. 4, the effect of small concentrations of Ca^{2+} (in per cent) in the presence of ATP and Mg^{2+} was more pronounced at pH 8.1 than at pH 7.6. Thus, with optimal concentrations of ATP, Mg^{2+}, and Ca^{2+} (which did not affect tryptophan hydroxylase activity under standard conditions, i.e., 10 or 50 μM), tryptophan hydroxylase activity doubled at pH 8.1, whereas it increased by only 72% at pH 7.6.

In another set of experiments, the effect of 10 μM Ca^{2+} was analyzed on tryptophan hydroxylase previously activated by ATP and Mg^{2+} and then filtered through a Sephadex G-25 column. Ca^{2+} failed to activate tryptophan hydroxylase under these conditions (Table 5). In fact, Ca^{2+} potentiated the stimulatory effect of ATP and Mg^{2+} only when it was added with these two compounds during the preliminary incubation period or the enzyme assay.

Although tryptophan hydroxylase was

TABLE 5

Effects of Ca^{2+} on tryptophan hydroxylase activity during or following incubation with ATP and Mg^{2+}

The 35,000 × *g* supernatant was first incubated for 7 min at 30° in the absence and presence of ATP (0.5 mM), magnesium acetate (5 mM), and calcium chloride (10 μM). It was then filtered through a Sephadex G-25 column and used as the enzyme source. Tryptophan hydroxylase was assayed at pH 8.1 with 0.2 mM tryptophan, 0.16 mM 6-MPH_4, and ATP (0.5 mM), magnesium acetate (5 mM), and/or calcium chloride (10 μM) as indicated. NaF (12.5 mM) was included in the prior incubation and assay mixtures. Each value is the mean of triplicate determinations.

Additions to medium before Sephadex chromatography	Additions to assay mixture	Tryptophan hydroxylase activity
		nmoles 5-HTP/mg protein/15 min (% control)
None	None	1.82 (100)
	Ca^{2+}	1.93 (106)
	ATP, Mg^{2+}	2.83 (155)
	ATP, Mg^{2+}, Ca^{2+}	4.07 (224)
ATP, Mg^{2+}	None	2.47 (100)
	Ca^{2+}	2.59 (105)
	ATP, Mg^{2+}	3.42 (138)
	ATP, Mg^{2+}, Ca^{2+}	4.25 (172)
ATP, Mg^{2+}, Ca^{2+}	None	4.10 (100)
	Ca^{2+}	4.11 (100)
	ATP, Mg^{2+}	4.57 (111)
	ATP, Mg^{2+}, Ca^{2+}	5.41 (132)

already activated during the prior incubation period with ATP, Mg^{2+}, and Ca^{2+}, the addition of these compounds to the assay mixture further increased the enzymatic activity (Table 5). This might indicate that the activation induced by phosphorylating conditions was reversible (the dephosphorylation reaction being not completely prevented by 12.5 mM NaF?) and that during the filtration procedure a significant part of this effect disappeared. The addition of ATP, Mg^{2+}, and Ca^{2+} to the assay mixture would then activate the enzyme back to its maximal level.

As expected, the addition of EGTA instead of Ca^{2+} to the tryptophan hydroxylase assay mixture resulted in a marked decrease in the stimulation of enzymatic activity by ATP and Mg^{2+} (Fig. 4). Replacement of ATP with App(NH)p greatly reduced the effect of 10 μM Ca^{2+} on tryptophan hydroxylase activity (Table 6). Adenosine slightly inhibited the activation of tryptophan hydroxylase occurring when ATP, Mg^{2+}, and 10 μM Ca^{2+} were included in the assay mixture (Table 6). In contrast, diphenylhydantoin, which inhibits a Ca^{2+}-dependent protein kinase in brain tissues (23), remained ineffective in this respect (Table 6).

Changes in kinetic parameters of tryptophan hydroxylase elicited by ATP, Mg^{2+} and Ca^{2+}. The activation of tryptophan hydroxylase (in the 35,000 × *g* supernatant) induced by ATP, Mg^{2+}, and Ca^{2+} was related mainly to increased apparent affinities of the enzyme for both tryptophan and 6-MPH_4 (Table 7). The slight increase in the V_{max} remained too small to be significant.

Using a semipurified preparation of tryptophan hydroxylase, Ca^{2+} was still found to potentiate slightly (15%) the effects of ATP and Mg^{2+}. The addition of ATP, Mg^{2+}, and Ca^{2+} to the assay mixture induced mainly a decrease in the apparent

TABLE 6

Effects of adenosine, diphenylhydantoin, and App(NH)p on tryptophan hydroxylase activity in the absence and presence of Mg^{2+}, Ca^{2+}, and/or ATP

Tryptophan hydroxylase activity was measured in the 35,000 × *g* supernatant at pH 8.1 with 0.2 mM tryptophan and 0.16 mM 6-MPH_4. Adenosine (1 mM), diphenylhydantoin (2.5 mM), App(NH)p (0.5 mM), ATP (0.5 mM), magnesium acetate (5 mM), and calcium chloride (10 μM) were added directly to the assay mixture as indicated. Each value is the mean of triplicate determinations.

Additions	Tryptophan hydroxylase activity
	nmoles 5-HTP/mg protein/15 min (% control)
None	2.16 (100)
ATP, Mg^{2+}, Ca^{2+}	3.84 (178)
Adenosine	1.78 (100)
Adenosine, ATP, Mg^{2+}, Ca^{2+}	2.86 (161)
Diphenylhydantoin	1.78 (100)
Diphenylhydantoin, ATP, Mg^{2+}, Ca^{2+}	3.11 (175)
App(NH)p	1.90 (100)
App(NH)p, Mg^{2+}, Ca^{2+}	2.26 (119)

TABLE 7

Kinetic parameters of tryptophan hydroxylase assayed in the absence and presence of ATP (0.5 mM), Mg^{2+} (5 mM), and Ca^{2} (10 μM)

The enzyme source was the 35,000 × *g* supernatant of a rat brain stem homogenate. The apparent K_m and V_{max} values were determined by linear regression analysis of double-reciprocal plots (see METHODS). Each value is the mean ± standard error. The number of experiments is indicated in parentheses.

Conditions	Tryptophan		6-MPH_4	
	K_m	V_{max}	K_m	V_{max}
	μM	*nmoles 5-HTP/mg protein/hr*	μM	*nmoles 5-HTP/mg protein/hr*
Standard assay	228 ± 11 (20)	23.7 ± 1.3	208 ± 14 (16)	26.7 ± 1.9
ATP, Mg^{2+}, Ca^{2+}	133 ± 7[a] (5)	28.4 ± 2.7	133 ± 14[a] (3)	31.8 ± 3.9

[a] $p < 0.05$ compared with control.

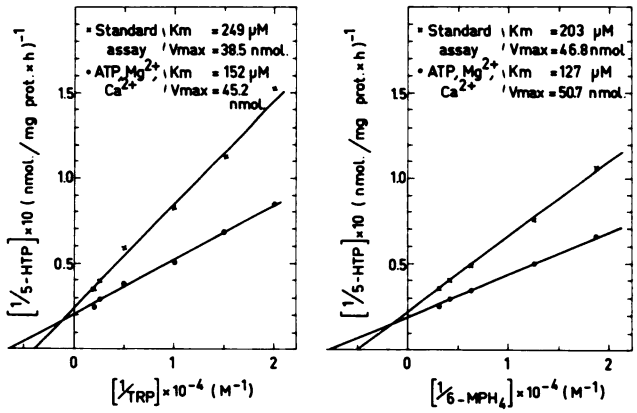

FIG. 5. *Lineweaver-Burk plots of tryptophan hydroxylase activity vs. tryptophan (TRP) or 6-MPH_4 concentration in the absence and presence of 0.5 mM ATP, 5 mM Mg^{2+}, and 50 μM Ca^{2+}*

The enzyme source was the semipurified preparation obtained by ammonium sulfate fractionation and Sephadex G-200 gel filtration. Assays were performed in 0.05 M Tris-acetate buffer, pH 7.6. Each point is the mean of triplicate determinations performed as described in METHODS.

K_m values of the semipurified tryptophan hydroxylase for both tryptophan and 6-MPH_4 (Fig. 5).

DISCUSSION

Phenylalanine, tyrosine, and tryptophan hydroxylases share many properties. The three enzymes can be activated by detergents like sodium dodecyl sulfate (24–26), by phospholipids (24, 27, 28), and by proteolytic enzymes (9, 29, 30). Recently several authors (14–16, 31) demonstrated that tyrosine hydroxylase can be activated under phosphorylating conditions. The same conclusion was also reported for hepatic phenylalanine hydroxylase (32). Since NaF (80 mM) was found to activate tryptophan hydroxylase by 26%[2] in a crude extract (35,000 × *g* supernatant of a rat brain stem homogenate), we first hypothesized that this was related to the well-known inhibitory effects of NaF towards phosphoprotein phosphatases (22). Teichberg and Changeux (33) used similar reasoning about the alteration of the acetylcholine receptor induced by NaF and subsequently demonstrated that a phosphorylation reaction was actually involved in this effect (34). In the case of tryptophan hydroxylase, the present results also indicate that, like NaF, the phosphorylation of proteins can induce the activation of this enzyme. Thus ATP and Mg^{2+} in the assay mixture (or during preliminary incubation) stimulated tryptophan hydroxylase activity. This effect was largely prevented by the addition of adenosine, a

[2] Unpublished observations.

potent protein kinase inhibitor (22). In addition, when App(NH)p (the terminal phosphate of which cannot be utilized for protein phosphorylation) replaced ATP in the assay mixture, no activation occurred.

However, there are striking differences among the three enzymes. For example, cyclic AMP is absolutely required for the activation of phenylalanine hydroxylase (32). In the case of tyrosine hydroxylase, significant activation occurs with ATP and Mg^{2+} alone (14, 16, 31), but the addition of cyclic AMP is necessary for maximal activation. The present data established that tryptophan hydroxylase, in the presence of ATP and Mg^{2+}, was not further activated by cyclic AMP. This would suggest that the endogenous protein kinase involved in the activation of tryptophan hydroxylase was not dependent upon cyclic AMP. Instead of a cyclic nucleotide, Ca^{2+} in low concentrations (5–50 μM) was found to potentiate the stimulatory effect of ATP and Mg^{2+}. This effect of Ca^{2+} was not exerted directly on the tryptophan hydroxylase, since in this range of concentrations the cation altered neither the activity of the native enzyme nor that of the Sephadex G-25-eluted enzyme previously activated by incubation with ATP and Mg^{2+}. In fact, the Ca^{2+} effect occurred only in the presence of ATP and Mg^{2+}, suggesting that it could be involved in a phosphorylation reaction. Indeed, several authors (35–39) have demonstrated that protein kinases could be activated by Ca^{2+}, notably in nerve terminals (37, 39). In this respect, it should be emphasized that Ca^{2+}-dependent protein kinases in the brain (36, 37) required Ca^{2+} concentrations in the same range as those shown to stimulate tryptophan hydroxylase activity in the presence of ATP and Mg^{2+}. Whatever this kinase may be, it cannot be the Ca^{2+}-dependent protein kinase recently characterized by DeLorenzo (37), since this enzyme was inhibited by diphenylhydantoin (23), in contrast to the enzyme possibly involved in the activation of tryptophan hydroxylase.

If the kinase responsible for the activation of tryptophan hydroxylase is not a cyclic AMP-dependent enzyme, it remains to be explained why the addition of a cyclic AMP-dependent protein kinase led to activation of this hydroxylase under some conditions. Recent studies (40) have revealed that the substrates of protein kinases exhibit very similar amino acid sequences surrounding the phosphorylated sites. Therefore the substrate specificity of these enzymes might be broad enough to explain why a cyclic AMP-dependent protein kinase could be used instead of the (Ca^{2+}-dependent) endogenous protein kinase in the activation of tryptophan hydroxylase.

In the case of phenylalanine hydroxylase (32) and tyrosine hydroxylase (41), the enzymatic activation induced by phosphorylating conditions probably resulted from phosphorylation of the hydroxylase itself. This conclusion concerning tyrosine hydroxylase was challenged, and it has been proposed (14, 15) that a protein other than the hydroxylase itself is phosphorylated in the presence of ATP, Mg^{2+}, and cyclic AMP. The partial purification of tryptophan hydroxylase did not suppress the activation by phosphorylating conditions. This suggests that the protein(s) which should be phosphorylated to induce the activation of tryptophan hydroxylase was not eliminated from the enzyme solution by the purification procedures used here. Moreover, the addition of phosphoproteins such as α-casein, phosvitin, and vitellin to the assay mixture did not change the activity of tryptophan hydroxylase (28). This indicates that a specific protein should be phosphorylated to induce the activation of tryptophan hydroxylase. All these data would suggest that the enzyme itself is phosphorylated. Indeed, evidence that tryptophan hydroxylase is likely a phosphoprotein *in vivo* was recently obtained by Gál.[3]

Kinetic analyses revealed that the apparent affinities of tryptophan hydroxylase for both tryptophan and its pterin cofactor were increased under phosphorylating conditions. In this respect tryptophan hydroxylase closely resembles tyrosine hydroxylase (12, 13) but not phenyl-

[3] E. M. Gál, personal communication.

alanine hydroxylase (32).

Another modification in tryptophan hydroxylase characteristics when ATP and Mg^{2+} were included in the assay mixture was displacement of its optimal pH from 7.6 to 7.9. Similarly, Lloyd and Kaufman (15) have noted that the optimal pH of bovine caudate tyrosine hydroxylase was increased from 6.0 to 7.4 when the enzyme was activated by phosphorylating conditions. However, when ATP and Mg^{2+} were removed from the solution of activated tryptophan hydroxylase, this shift in optimal pH was no longer detected. As already discussed by Goldstein *et al.* (16), the pH effect might be related only to the phosphorylation reaction, so that in the absence of ATP and Mg^{2+} it did not occur. Indeed, in the range pH 8–8.5 phosphorylation might be optimal, whereas at lower pH values the dephosphorylation reaction would be rapid enough to reverse the effect induced by slower phosphorylation.

At present the activation of tryptophan hydroxylase by phosphorylation under physiological conditions is still an open question. Although further experiments are required to solve this problem, it should be emphasized that the Ca^{2+} concentrations required for maximal activation were in the physiological range. This is in sharp contrast with the activating effect of Ca^{2+}-dependent proteolysis, which requires millimolar concentrations of the cation (9).

ACKNOWLEDGMENTS

The authors with to thank Dr. J. Glowinski for continuous advice and encouragement.

REFERENCES

1. Eccleston, D., Ritchie, I. M. & Roberts, M. H. T. (1970) *Nature*, 226, 84–85.
2. Shields, P. J. & Eccleston, D. (1972) *J. Neurochem.*, 19, 265–272.
3. Herr, B. E., Gallager, D. W. & Roth, R. H. (1975) *Biochem. Pharmacol.*, 24, 2019–2023.
4. Carlsson, A., Bedard, P., Lindqvist, M. & Magnusson, T. (1972) *Biochem. Soc. Symp.*, 36, 17–32.
5. Carlsson, A., Lindqvist, M., Magnusson, T. & Atack, C. (1973) *Naunyn-Schmiedebergs Arch. Pharmacol.*, 277, 1–12.
6. Herr, B. E. & Roth, R. H. (1976) *Brain Res.*, 110, 189–193.
7. Boadle-Biber, M. C. (1975) *Biochem. Pharmacol.*, 24, 1455–1460.
8. Knapp, S., Mandell, A. J. & Bullard, W. P. (1975) *Life Sci.*, 16, 1583–1594.
9. Hamon, M., Bourgoin, S., Artaud, F. & Héry, F. (1977) *J. Neurochem.*, 28, 811–818.
10. Morgenroth, V. H., III, Boadle-Biber, M. C. & Roth, R. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, 71, 4283–4287.
11. Weiner, N., Lee, F. L., Barnes, E. & Dreyer, E. (1977) in *Biochemistry and Function of Monoamine Enzymes* (Usdin, E., Weiner, N. & Youdim, M. B. H., eds.), Marcel Dekker, New York, in press.
12. Roth, R. H., Morgenroth, V. H., III & Salzman, P. M. (1975) *Naunyn-Schmiedebergs Arch. Pharmacol.*, 289, 327–343.
13. Murrin, L. C., Morgenroth, V. H., III & Roth, R. H. (1976) *Mol. Pharmacol.*, 12, 1070–1081.
14. Lovenberg, W., Bruckwick, E. A. & Hanbauer, I. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, 72, 2955–2958.
15. Lloyd, T. & Kaufman, S. (1975) *Biochem. Biophys. Res. Commun.*, 66, 907–913.
16. Goldstein, M., Bronaugh, R. L., Ebstein, B. & Roberge, C. (1976) *Brain Res.*, 109, 563–574.
17. Gál, E. M. & Patterson, K. (1973) *Anal. Biochem.*, 52, 625–629.
18. Tachiki, K. H. & Aprison, M. H. (1975) *Anal. Chem.*, 47, 7–11.
19. Waalkes, T. P. & Udenfriend, S. (1957) *J. Lab. Clin. Med.*, 50, 733–736.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
21. Snedecor, G. W. & Cochran, W. G. (1967) *Statistical Methods*, Iowa State University Press, Ames.
22. Miyamoto, E., Kuo, J. F. & Greengard, P. (1969) *J. Biol. Chem.*, 244, 6395–6402
23. DeLorenzo, R. J. & Glaser, G. H. (1976) *Brain Res.*, 105, 381–386.
24. Fisher, D. B. & Kaufman, S. (1972) *J. Biol. Chem.*, 247, 2250–2252.
25. Kuczenski, R. (1974) *Life Sci.*, 14, 2379–2384.
26. Hamon, M., Bourgoin, S., Héry, F., Ternaux, J. P. & Glowinski, J. (1976) *Nature*, 260, 61–63.
27. Lloyd, T. & Kaufman, S. (1974) *Biochem. Biophys. Res. Commun.*, 59, 1262–1269.
28. Hamon, M., Bourgoin, S., Héry, F. & Glowinski, J. (1977) in *Biochemistry and Function of Monoamine Enzymes* (Usdin, E., Weiner, N. & Youdim, M. B. H., eds.), Marcel Dekker, New York, in press.
29. Fisher, D. B. & Kaufman, S. (1973) *J. Biol. Chem.*, 248, 4345–4353.

30. Kuczenski, R. (1973) *J. Biol. Chem.*, 248, 2260–2265.
31. Morgenroth, V. H., III, Hegstrand, L. R., Roth, R. H. & Greengard, P. (1975) *J. Biol. Chem.*, 250, 1946–1948.
32. Milstien, S., Abita, J. P., Chang, N. & Kaufman, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, 73, 1591–1593.
33. Teichberg, V. I. & Changeux, J.-P. (1976) *FEBS Lett.*, 67, 264–268.
34. Teichberg, V. I. & Changeux, J.-P. (1977) *FEBS Lett.*, 74, 71–76.
35. Krebs, E. G., Stull, J. T., England, P. J., Huang, T. S., Brostrom, C. O. & Vandenheede, J. R. (1973) in *Protein Phosphorylation in Control Mechanisms* (Huijing, F. & Lee, E. Y. C., eds.), pp. 31–45, Academic Press, New York.
36. Ozawa, E. (1973) *J. Neurochem.*, 20, 1487–1488.
37. DeLorenzo, R. J. (1976) *Biochem. Biophys. Res. Commun.*, 71, 590–597.
38. Chacko, S., Conti, M. A. & Adelstein, R. S. (1977) *Proc. Natl. Acad. Sci. U. S. A.*, 74, 129–133.
39. Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.*, 252, 2764–2773.
40. Williams, R. E. (1976) *Science*, 192, 473–474.
41. Raese, J. D., Edleman, A. M., Lazar, M. A. & Barchas, J. D. (1977) in *Biochemistry and Function of Monoamine Enzymes* (Usdin, E., Weiner, N. & Youdim, M. B. H., eds.), Marcel Dekker, New York, in press.