Involvement of activator protein in the activation of tryptophan hydroxylase by cAMP-dependent protein kinase

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The tryptophan hydroxylase activity of the crude extract from rat brain stem was stimulated approximately 2-fold by incubation with cAMP analogues under protein phosphorylating conditions. The cAMP-dependent activation process of the enzyme needed not only cAMP-dependent protein kinase but also activator protein. The kinetic properties of the enzyme activated by cAMP-dependent protein kinase were very similar to those of the enzyme activated by calmodulin-dependent protein kinase II.

Tryptophan hydroxylase; cAMP-dependent protein kinase; Calmodulin-dependent protein kinase II; Activator protein; 14-3-3 protein; Rat brain

1. INTRODUCTION

Tryptophan hydroxylase [1,2] and tyrosine hydroxylase [3,4] catalyze the initial and probably rate-limiting steps in the biosynthesis of serotonin and catecholamines, respectively, in the central nervous system, and therefore the regulation of both enzymes is of great importance. Tyrosine hydroxylase is activated through phosphorylation by PKA [5-7] and CaM-kinase II [8] by quite distinct mechanisms [9,10]. In contrast to tyrosine hydroxylase, the effect of cAMP-dependent phosphorylation on tryptophan hydroxylase activity remains unsettled. The activity of tryptophan hydroxylase of rat brainstem slices was reported to be stimulated 2-fold by cAMP analogues [11], but such a remarkable activation was not observed with soluble brain extracts [12,13]. Thus, conflicting results have been reported about the activation of tryptophan hydroxylase by the cAMP-dependent system.

The present study demonstrated that tryptophan hydroxylase from rat brainstem was activated by PKA and that the activation required activator protein (14-3-3 protein [14]), which is required for the activation of both tryptophan hydroxylase and tyrosine hydroxylase by CaM-kinase II [8]. Some kinetic properties of the enzyme activated by PKA and that activated by CaM-kinase II were also examined for comparison.

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Abbreviations: PKA, cAMP-dependent protein kinase; CaM-kinase II, calmodulin-dependent protein kinase II; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NSD-1015, 3-hydroxybenzylhydrazine; $6MPH_4$, 2-amino-4-hydroxy-6-methyltetrahydropteridine; 8Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; db-cAMP, N^6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate

2. MATERIALS AND METHODS

Catalase, phosphoenolpyruvate and pyruvate kinase were from Boehringer. Dithiothreitol, Mes and Hepes were from Sigma. NSD-1015 was from Aldrich. $6MPH_4$ was from Calbiochem. Antipain, pepstatin 4, chymostatin and leupeptin were from Peptide Institute, Osaka, Japan.

The crude extract of rat brainstem was prepared as described previously [15]. Partially purified tryptophan hydroxylase was obtained by pteridine-affinity chromatography as described previously [16], except for omission of catalase from the buffers used. The catalytic subunit of PKA was purified from bovine heart [17]. CaM-kinase II [18] and activator protein [8] were purified from rat cerebral cortex. Calmodulin was purified from rat testis [19].

Tryptophan hydroxylase was assayed by HPLC-fluorometric detection [20]. The standard assay mixture (100 μl) contained 50 mM Hepes-NaOH (pH 7.2), 400 μM L-tryptophan, 200 μM $6MPH_4$, 2 mM dithiothreitol, 40 μM ferrous ammonium sulfate, 40 μg of catalase, 50 μM NSD-1015 and a suitable amount of the enzyme. The reaction was carried out at 30°C for 5 min with shaking.

When the enzyme was measured after exposure to enzymatic phosphorylating conditions, the incubation mixture (200 μl) for phosphorylation of the enzyme in the crude extract contained 50 mM Hepes-NaOH (pH 7.2), 5 μg/ml each of antipain, pepstatin A, chymostatin and leupeptin, 10 mM NaF, 5 mM magnesium acetate, 0.1 mM ATP, 3 mM phosphoenolpyruvate (pH 7), 20 μg of pyruvate kinase and a suitable amount of the crude extract. For phosphorylation of the partially purified enzyme by PKA, the mixture (50 μl) contained 50 mM Hepes-NaOH (pH 7.2), 0.5 mM ATP, 5 mM magnesium acetate, 2 mM dithiothreitol, 1.2 μg of the catalytic subunit of PKA and a suitable amount of the enzyme. For the phosphorylation by CaM-kinase II, the catalytic subunit of PKA was replaced by 0.24 μg of CaM-kinase II, 0.36 μg of calmodulin, and 0.1 mM $CaCl_2$. After incubation for 5 min at 30°C, tryptophan hydroxylase activity was measured in the presence of 4.6 μg/ml of activator protein. One unit of the activity of tryptophan hydroxylase

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was defined as the amount of the enzyme that catalyzed the formation of 1 pmol of 5-hydroxytryptophan/min at 30°C.

Protein was determined with bovine serum albumin as a standard [21].

3. RESULTS AND DISCUSSION

When the crude extract of the rat brainstem was incubated with 8Br-cAMP or db-cAMP at 30°C under protein phosphorylating conditions, the activity of tryptophan hydroxylase increased gradually as shown in Fig. 1, indicating that tryptophan hydroxylase might be activated by PKA. In contrast to the above observation with the crude extract, the incubation of the partially purified tryptophan hydroxylase with the catalytic subunit of PKA failed to activate the enzyme, suggesting the involvement of something present in the crude extract in the activation of the enzyme by PKA. Fig. 2A shows that activator protein, which is known to be involved in the activation of tryptophan hydroxylase by CaM-kinase II [8], was also involved in the activation by PKA. The concentration of activator protein required for half-maximal activation of the enzyme was 0.5–1.0 μg/ml, in agreement with that reported in the activation of the enzyme by CaM-kinase II [8]. The activation of tryptophan hydroxylase absolutely required the presence of PKA (Fig. 2B) as well as activator protein (Fig. 2A) and the activation was dose dependent. The activation increased as incubation time increased, finally reaching a maximum level, and also it increased with an increase in incubation temperature, as shown in Fig. 3. Thus, PKA appears to be involved in the activation of tryptophan hydroxylase in cooperation with activator protein.

Fig. 4 shows the pH profiles of the activities of the enzymes activated by PKA, CaM-kinase II and both. The activities of the three preparations were very similar, and no additive effects of PKA and CaM-kinase II were observed, suggesting that the enzyme might be activated in a similar manner by PKA and by CaM-kinase II. Table II shows the kinetic parameters of the enzyme activated by PKA and by CaM-kinase II together with those of the original unactivated enzyme. The two protein kinases showed similar effects on the kinetic parameters, resulting in no change in the K_m

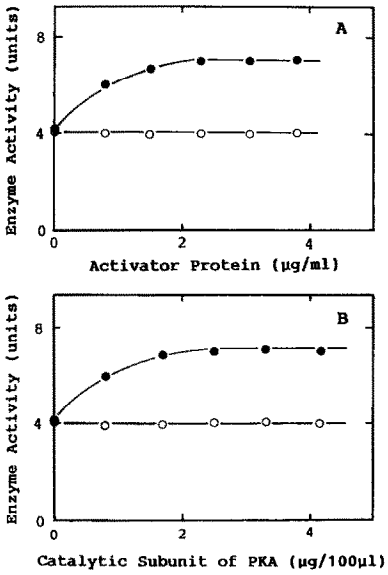

Fig. 2. Effects of varying the concentrations of activator protein and catalytic subunit of PKA on the activation of tryptophan hydroxylase. Purified tryptophan hydroxylase was incubated with 5 mM magnesium acetate, 2.4 μg of the catalytic subunit of PKA and different amounts of activator protein (A) and incubated with 5 mM magnesium acetate, 4.6 μg/ml of activator protein and different amounts of the catalytic subunit of PKA (B), in the absence (○) and presence (●) of ATP under the standard assay conditions.

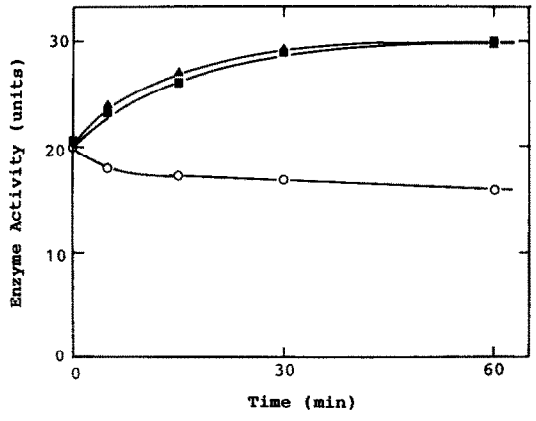

Fig. 1. Time course of the activation of tryptophan hydroxylase in the crude extract of rat brainstem by cAMP analogues under phosphorylating conditions. The crude extract was incubated under phosphorylating conditions as described in section 2 with the following additions: none (○), 0.5 mM 8Br-cAMP (▲) and 0.5 mM db-cAMP (■). At the indicated times, 30-μl aliquots were assayed for tryptophan hydroxylase.

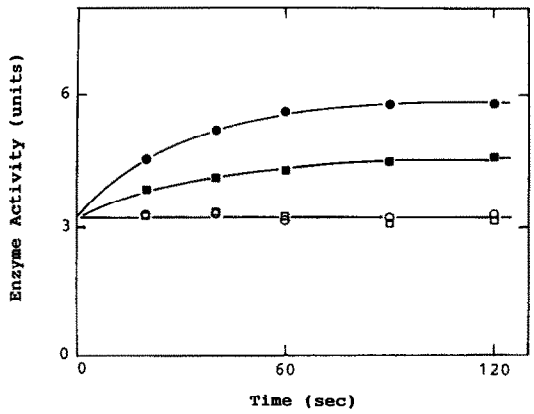

Fig. 3. Time course of the activation of tryptophan hydroxylase by PKA. Purified tryptophan hydroxylase was incubated with 5 mM magnesium acetate and 1.0 μg of the catalytic subunit of PKA in the absence (○) and presence (●) of ATP at 30°C and in the absence (□) and presence (■) of ATP at 20°C. At the indicated times, tryptophan hydroxylase activity was assayed for 2 min as described in section 2.

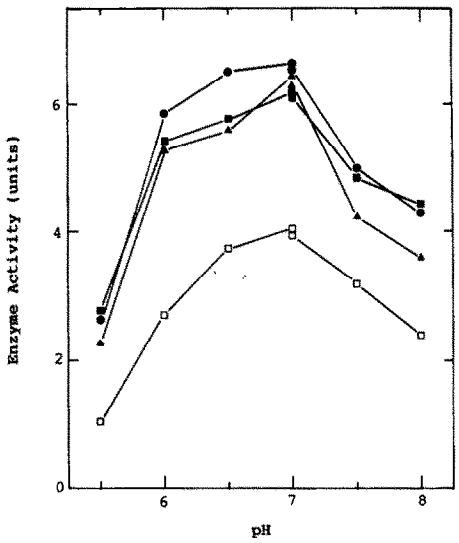

Fig. 4. Effects of PKA, CaM-kinase II and both kinases on pH dependence of tryptophan hydroxylase activity. Purified tryptophan hydroxylase was incubated under phosphorylating conditions as described in section 2 with the following additions: none (□), the catalytic subunit of PKA (●), CaM-kinase II (■) and both kinases (▲) and the activity was assayed as described in section 2, except that 0.1 M Mes (pH 5.5–7.0) and 0.1 M Hepes (pH 7.0–8.0) were used in place of 50 mM Hepes (pH 7.2).

value for L-tryptophan, in a slight decrease in the K_m for $6MPH_4$, and in an increase in the V_{max} of the enzyme. Thus, tryptophan hydroxylase appears to be activated in a similar manner by PKA and by CaM-kinase II, and the activation may be due to an increase in both the affinity for the cofactor and the V_{max} of the enzyme.

Tryptophan, tyrosine and phenylalanine hydroxylases require a reduced pterin cofactor as an electron donor for their catalytic reactions and share a number of properties [22]. Among them, tyrosine and phenylalanine hydroxylases have so far been reported to be activated by mechanisms in which PKA is involved and in which CaM-kinase II is involved. Tyrosine hydroxylase is activated in quite a different manner by PKA and by CaM-kinase II. In contrast, phenylalanine hydroxylase is activated by the two kinases in a similar manner, and both the kinases have been demonstrated to phosphorylate the same site of the enzyme [23]. The phosphorylation sites of tryptophan hydroxylase by the two kinases remain to be studied.

It has recently been reported that the deduced amino acid sequence of protein kinase C inhibitor protein from sheep brain is highly homologous to that of activator protein [24], providing some insight into the significance of activator protein in the functioning of the central nervous system.

Table I

Comparison of apparent kinetic parameters of tryptophan hydroxylase

Protein kinase added	K_m (μM)		V_{max} (pmol/min)
	L-Trp	$6MPH_4$	
None	87	119	7
PKA	87	80	11
CaM-kinase II	87	74	11

After purified tryptophan hydroxylase was incubated with the indicated protein kinase under phosphorylating conditions, the activity was measured as described in section 2, except that the concentrations of L-tryptophan and $6MPH_4$ were varied. The apparent K_m values for L-tryptophan and $6MPH_4$ were determined from double-reciprocal plots in the presence of 1 mM $6MPH_4$ and 1 mM L-tryptophan, respectively

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