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TYROSINE 3-MONOOXYGENASE IS PHOSPHORYLATED BY Ca^{2+}-, CALMODULIN-DEPENDENT PROTEIN KINASE, FOLLOWED BY ACTIVATION BY ACTIVATOR PROTEIN

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SUMMARY: The activation of tyrosine 3-monooxygenase by Ca^{2+}-calmodulin-dependent protein kinase in the presence of Ca^{2+}, calmodulin, and ATP required the presence of activator protein. When tyrosine 3-monooxygenase was incubated with [γ-^{32}P]ATP and Ca^{2+}-,calmodulin-dependent protein kinase in the absence of the activator protein, the enzyme was phosphorylated but not activated. When the activator protein was added to the phosphorylated tyrosine 3-monooxygenase, the enzyme was activated. Thus, the activation of tyrosine 3-monooxygenase occurred in a two step reaction, phosphorylation by Ca^{2+}-,calmodulin-dependent protein kinase and activation by activator protein.

INTRODUCTION

We have recently demonstrated that tyrosine 3-monooxygenase and tryptophan 5-monooxygenase, the rate-limiting enzymes in the biosynthesis of catecholamines and serotonin, respectively, are regulated by Ca^{2+}-,calmodulin-dependent protein kinase (1,2) and the activation of both enzymes required not only the protein kinase but also the activator protein in the presence of ATP, Mg^{2+}, Ca^{2+} and calmodulin (3).

The present studies demonstrate that the phosphorylation and the activation of tyrosine 3-monooxygenase were distinct reactions and that tyrosine 3-monooxygenase was phosphorylated by

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Abbreviations: 6-MPH_4, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morphorino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

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Ca^{2+}-,calmodulin-dependent protein kinase, followed by activation by activator protein.

EXPERIMENTAL PROCEDURES

Materials——[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Radiochemical Centre, Amersham. 3-Isobutyl-1-methylxanthine was from Aldrich.

Rabbit antiserum against bovine adrenal tyrosine 3-monooxygenase was prepared as described previously (4).

Enzyme Purification——Tyrosine 3-monooxygenase was prepared from bovine adrenal medulla as described previously (5) through the heparin-Sepharose step. Ca^{2+}-,calmodulin-dependent protein kinase was prepared from rat cerebral cortex as described previously (6) through the calmodulin-Sepharose step. Activator protein was purified from rat cerebral cortex as described previously (3). cAMP-dependent protein kinase from bovine heart was prepared by the method of Rubin *et al.* (7) without treatment of almina Cγ-gel. The enzyme was purified about 300-fold and free from activator protein. Rat brain calmodulin was prepared by the method of Wang and Desai (8).

Tyrosine 3-Monooxygenase Assay——Tyrosine 3-monooxygenase was assayed fluorometrically (9). When the enzyme activity was measured after incubation under activating conditions, the assay medium contained 100 mM Mes buffer, pH 6.5, 0.2 mM tyrosine, 0.1 mM 6-MPH_4, 40 mM 2-mercaptoethanol, 2 mM EDTA, 20 mM NaF, 0.1 mg of catalase and a suitable amount of tyrosine 3-monooxygenase in a final volume of 0.5 ml. The reaction was carried out at 30 °C for 10 min with shaking. One unit of tyrosine 3-monooxygenase is defined as the amount which catalyzes the formation of 1 nmol of 3,4-dihydroxyphenylalanine (Dopa) per min at 30 °C.

Activation of Tyrosine 3-Monooxygenase by Protein Kinases—— The standard preincubation mixture for the activation of tyrosine 3-monooxygenase contained 50 mM Hepes buffer, pH 7.0, 0.5 mM ATP, 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 20 mM NaF, 1.5 mM 3-isobutyl-1-methylxanthine, and a suitable amount of tyrosine 3-monooxygenase. For the activation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase, 0.12 mM $CaCl_2$, calmodulin, activator protein and Ca^{2+}-,calmodulin-dependent protein kinase were added to the standard preincubation mixture. For the activation of tyrosine 3-monooxygenase by cAMP-dependent protein kinase, 0.01 mM cAMP and cAMP-dependent protein kinase were added to the standard preincubation mixture. The mixture was incubated at 30 °C for 5 min and then the activity of tyrosine 3-monooxygenase was assayed as described above.

Phosphorylation of Tyrosine 3-Monooxygenase——Phosphorylation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase was carried out under the conditions used for the activation of the enzyme, except that 0.5 mM ATP was replaced by 0.1 mM [γ-^{32}P]ATP. The incorporation of [^{32}P]phosphate into tyrosine 3-monooxygenase was analyzed by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis as described previously (4).

SDS-Polyacrylamide Gel Electrophoresis——SDS-polyacrylaimde gel electrophoresis was carried out with 10% polyacrylamide gels in the presence of 0.1% SDS at 8 mA/gel for 5 h according to the procedure of Weber and Osborn (10).

Protein Determination——Protein concentrations were estimated

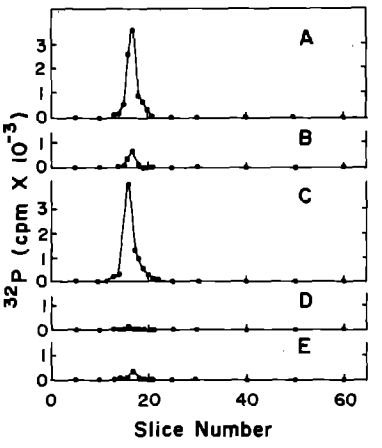

Fig. 1. Requirements for phosphorylation of tyrosine 3-monooxygenase. (*A*), complete system contained 50 mM Hepes buffer, pH 7.0, 0.1 mM [γ-^{32}P]ATP (8 X 10^5 cpm), 5 mM $Mg(CH_3COO)_2$, 0.12 mM $CaCl_2$, 0.1 mM EGTA, 20 mM NaF, 3.7 µg of activator protein, 5 µg of calmodulin, 11 µg of Ca^{2+}-,calmodulin-dependent protein kinase and 28 µg of tyrosine 3-monooxygenase in a final volume of 0.1 ml. (*B*), Ca^{2+}-,calmodulin-dependent protein kinase was omitted from the complete system, (*C*), activator protein was omitted, (*D*), calmodulin was omitted, and (*E*), Ca^{2+} was omitted. After incubation for 5 min at 30 °C, 20 µl of specific antiserum to tyrosine 3-monooxygenase were added to the reaction mixture and immunoprecipitation was carried out as described under "Experimental Procedures." The immunoprecipitate was dissolved in 0.1 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 1% SDS, 10 % glycerol and 2% 2-mercaptoethanol. After heating for 3 min at 100 °C, the mixture was subjected to SDS-polyacrylamide gel electrophoresis and ^{32}P radioactivity was measured as described under "Experimental Procedures."

by the method of Lowry *et al.* (11) with bovine serum albumin as a standard.

RESULTS

We have recently reported that the activation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase in the presence of Ca^{2+}, calmodulin, and ATP required the presence of the activator protein (3). Fig. 1 shows, however, that the phosphorylation of tyrosine 3-monooxygenase required Ca^{2+}-, calmodulin-dependent protein kinase, calmodulin, and Ca^{2+} but did not require the presence of the activator protein. Thus, the phosphorylation of tyrosine 3-monooxygenase by Ca^{2+}-, calmodulin-dependent protein kinase occured in the absence of the

Table I. Activation of tyrosine 3-monooxygenase by Ca^{2+}-, calmodulin-dependent protein kinase and activator protein

Time of addition		Tyrosine 3-monooxygenase	
- 5 min (Preincubation)	0 min (assay)	unit	%
None	None	0.484	100
Kinase	None	0.550	114
Activator	None	0.496	103
Kinase + Activator	None	0.985	202
Kinase	Activator	0.968	200
Activator	Kinase	0.484	100

Tyrosine 3-monooxygenase (11 μg of protein) was previously incubated at 30 °C for 5 min in the standard preincubation mixture containing 0.12 mM $CaCl_2$ and calmodulin (2 μg of protein) as described under "Experimental Procedures" and the reaction was started by the addition of 0.4 ml of the assay mixture for tyrosine 3-monooxygenase. Ca^{2+}-,calmodulin-dependent protein kinase (11 μg of protein) and activator protein (3.7 μg of protein) were added at the indicated times.

activator protein, suggesting that the activation and the phosphorylation of the enzyme may be distinct reactions.

To examine the relationship between the phosphorylation and the activation of tyrosine 3-monooxygenase, separation of both reactions was attempted as shown in Table I. Preincubation mixtures contained the ingredients necessary for the reaction of protein phosphorylation and therefore EDTA was added to the assay mixture to prevent phosphorylation of the enzyme during incubation as described under "Experimental Procedures." The preincubation of tyrosine 3-monooxygenase with either Ca^{2+}-,calmodulin-dependent protein kinase or activator protein was not significantly effective in activating the enzyme but the preincubation with both was effective, in accord with our previous observation (3),

suggesting that the phosphorylation of the enzyme by Ca^{2+}-, calmodulin-dependent protein kinase may be a necessary but not sufficient condition for the enzyme activation. When tyrosine protein kinase and then activator protein was added to the assay mixture, the enzyme was activated to the same extent as when the enzyme was preincubated with the kinase and activator protein, indicating that activator protein may act immediately on tyrosine 3-monooxygenase phosphorylated by the action of Ca^{2+}-,calmodulin-dependent protein kinase.

In contrast to the phosphorylation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase, the phosphorylation of the enzyme by cAMP-dependent protein kinase resulted in the activation of the enzyme without activator protein. No further activation was observed on incubation with the activator protein as shown in Table II. Thus, the activator protein was effective with the enzyme phosphorylated by Ca^{2+}-, calmodulin-dependent protein kinase but not effective with the enzyme phosphorylated by cAMP-dependent protein kinase and the non-phosphorylated enzyme.

DISCUSSION

The present studies demonstrated that the activation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase occurred in a two step reaction. The first step was the phosphorylation of the enzyme catalyzed by Ca^{2+}-,calmodulin-dependent protein kinase and the second step was the activation of the phosphorylated enzyme caused by the action of activator protein. Thus, the phosphorylation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase resulted in no significant change in the enzyme activity but appeared to cause a conformational change of the enzyme in such a way that the enzyme

Table II. Effects of activator protein on the activation of tyrosine 3-monooxygenase by Ca^{2+}-,calmodulin-dependent protein kinase or cAMP-dependent protein kinase

Experiments	Kinase added	Tyrosine 3-monooxygenase			
		+ Activator		- Activator	
		unit	(%)		
1	None	0.183	(100)	0.183	(100)
2	Ca^{2+}-,calmodulin-dependent	0.351	(193)	0.210	(115)
3	cAMP-dependent	0.426	(233)	0.413	(226)

Tyrosine 3-monooxygenase (3.8 μg of protein) was incubated in the standard preincubation mixture in the presence or absence of the activator protein (3 μg of protein) with the following additions; Experiment 1, 0.12 mM $CaCl_2$, 2 μg of calmodulin and 0.01 mM cAMP; Experiment 2, 0.12 mM $CaCl_2$, 2 μg of calmodulin and 9 μg of Ca^{2+}-, calmodulin-dependent protein kinase; Experiment 3, 0.01 mM cAMP and 3 μg of cAMP-dependent protein kinase. After 5 min incubation, 0.4 ml of the assay mixture for tyrosine 3-monooxygenase was added and the enzyme activity was measured under "Experimental Procedures."

became activated by the action of activator protein. Since the activation of tryptophan 5-monooxygenase required the presence of both Ca^{2+}-,calmodulin-dependent protein kinase and activator protein, as reported previously (3), it is reasonable to assume that tryptophan 5-monooxygenase is also activated by the two step reaction . Taking into account the fact that activator protein occurred abundantly in the cytosol fraction of a variety of tissues including brain tissue (3), the actual regulatory step may be the phosphorylation step through the action of Ca^{2+}-,calmodulin-dependent protein kinase. The physiological significance of the two step regulatory mechanism remains to be clarified. Similar two step mechanism has recently been reported in activation of phosphofructokinase in liver (12).

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