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ACTIVATION OF TRYPTOPHAN 5-MONOOXYGENASE BY CALCIUM-DEPENDENT REGULATOR PROTEIN

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SUMMARY: Rat brainstem tryptophan 5-monooxygenase was activated about 2-fold by rat brain calcium-dependent regulator (CDR) protein. The activation required both ATP and Mg^{2+} in the presence of low concentrations of Ca^{2+}.

Tryptophan 5-monooxygenase (EC 1.14.16.4) which catalyzes the conversion of tryptophan to 5-hydroxytryptophan is the rate limiting step in the biosynthesis of serotonin in brain (1,2). It has been reported that Ca^{2+} can activate tryptophan 5-monooxygenase in brain extracts (3-8). We have recently demonstrated the reversible activation of rat brainstem tryptophan 5-monooxygenase by incubation with ATP and Mg^{2+} in the presence of micromolar concentrations of Ca^{2+}(9). These results suggest that rat brainstem tryptophan 5-monooxygenase may be regulated by Ca^{2+}-dependent protein phosphorylation system (9).

The present studies demonstrated that rat brainstem tryptophan 5-monooxygenase can be activated by CDR in the presence of ATP, Mg^{2+}, and micromolar concentrations of Ca^{2+}. This observation together with our previous studies indicates that tryptophan 5-monooxygenase may be regulated by CDR-dependent protein kinase in rat brainstem.

Abbreviations: CDR, calcium-dependent regulator; 6-MPH_4, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

The brainstems of Wistar rats were homogenized in 50 mM Tris/acetate buffer, pH 7.6, containing 2 mM dithiothreitol with a Potter-Elvehjem homogenizer and centrifuged at 39,000 X g for 30 min. To the supernatant were added tryptophan and EGTA to final concentrations of 5 mM and 1 mM, respectively, and then a saturated ammonium sulfate solution (pH 7.6) was added to a final saturation of 25%. After the precipitate was removed by centrifugation, saturated ammonium sulfate solution (pH 7.6) was added to 55% saturation. The pellet was collected and dissolved in 20 mM Tris/acetate buffer, pH 7.6, containing 2 mM dithiothreitol and dialyzed against 100 volumes of the same buffer for 2 hr. This preparation (Fraction I) was used as the enzyme sourse.

Rat brain CDR was prepared by the method of Wang and Desai (10).

Tryptophan 5-monooxygenase assay was carried out essentially according to the procedure of Friedman et al. (11) as modified by Baumgarten et al. (12). In the standard assay for the activation of tryptophan 5-monooxygenase, the activation reaction was carried out simultaneously with tryptophan 5-monooxygenase reaction in order to prevent denaturation of the enzyme. The incubation mixture contained 50 mM Hepes buffer, pH 7.1, 500 μM ATP, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM EGTA, 3.5 μg of brain CDR, 400 μM tryptophan, 300 μM 6-MPH_4, 2 mM dithiothreitol, 50 μg of catalase and a suitable amount of tryptophan 5-monooxygenase in a final volume of 400 μl. The incubation was carried out at 30 °C for 20 min with shaking. The reaction was stopped by the addition of 40 μl of 60% perchloric acid. After precipitated protein was removed by centrifugation, 5-hydroxytryptophan was determined. One unit of tryptophan 5-monooxygenase is defined as the amount which catalyzes the formation of 1 nmol of 5-hydroxytryptophan per min at 30 °C.

Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as a standard.

Polyacrylamide disc gel electrophoresis was carried out at 4 °C with 7.5% polyacrylamide gels essentially according to the procedure of Davis (14).

RESULTS AND DISCUSSION

We have recently reported that tryptophan 5-monooxygenase in rat brainstem cytosol was activated about 2-fold by incubation with 0.5 mM ATP and 5 mM $MgCl_2$ in the presence of micromolar concentrations of Ca^{2+} (9). The fact that the activation was time-dependent and reversible suggested the possible participation of activating system, probably Ca^{2+}-dependent protein phosphorylation system, in the activation of tryptophan 5-monooxygenase (9). The resolution of tryptophan 5-monooxygenase activity and its activating activity was successfully carried

Table I. Effect of activator on activity of tryptophan 5-monooxygenase

Fraction	Tryptophan 5-monooxygenase activity unit	Activation (fold)
Fraction I	0.045	1
Fraction II	0.00	0
Fraction I + Fraction II	0.126	2.80
Fraction I + brain CDR	0.123	2.74
Fraction I + Fraction II + brain CDR	0.130	2.89

Fraction I, 25 to 55% saturated $(NH_4)_2SO_4$ fraction, and Fraction II, 55 to 90% saturated $(NH_4)_2SO_4$ fraction, were obtained from rat brainstem extract. Fraction I (423 μg of protein), Fraction II (123 μg of protein) and/or brain CDR (3.5 μg of protein) were incubated in a mixture containing 50 mM Hepes buffer, pH 7.1, 500 μM ATP, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM EGTA, 400 μM tryptophan, 300 μM 6-MPH_4, 2 mM dithiothreitol and 50 μg of catalase in a total volume of 400 μl at 30 °C for 20 min.

out by ammonium sulfate fractionation as shown in Table I. Fraction II exhibited no tryptophan 5-monooxygenase activity but tryptophan 5-monooxygenase activity of Fraction I was activated 2.5-fold by the addition of Fraction II in the presence of ATP, $MgCl_2$ and a low concentration of Ca^{2+}. Tryptophan 5-monooxygenase activity of Fraction I was not affected by incubation with ATP, Mg^{2+} and Ca^{2+} in the absence of Fraction II, indicating that Fraction I was completely depleted of the activator. When Fraction II was subjected to electrophoresis on 7.5% polyacrylamide gel, the single peak of the activating activity of tryptophan 5-monooxygenase was observed at the position corresponding to the bromophenol blue dye front as shown in Fig. 1. Since the purified CDR was known to have an electrophoretic mobility almost identical to that of the dye front on 7.5% polyacrylamide gels (15), the activation of tryptophan 5-monooxygenase by Fraction II was presumed to reflect

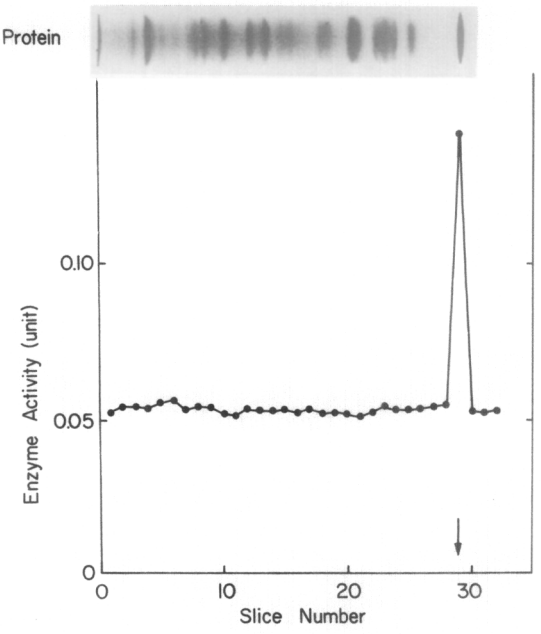

Fig. 1. Polyacrylamide gel electrophoresis of Fraction II. Fraction II (123 μg of protein) was subjected to electrophoresis on 7.5% polyacrylamide gel. After electrophoresis, the gel was sliced into 2-mm sections and was homogenized in 0.5 ml of assay mixture for tryptophan 5-monooxygenase consisting of 200 μl of water and 300 μl of the mixture containing 100 mM Hepes buffer, pH 7.1, 1 mM ATP, 10 mM $MgCl_2$, 200 μM $CaCl_2$, 200 μM EGTA, 800 μM tryptophan, 600 μM 6-MPH_4, 4 mM dithiothreitol and 100 μg of catalase. The homogenate was centrifuged at 3,000 rpm for 3 min in order to collect the gel in the bottom of a test tube, then, 100 μl of tryptophan 5-monooxygenase (Fraction I, 454 μg of protein) was added and the enzyme activity was assayed. Protein was visualized by Coomassie blue. The arrow indicates the position of dye.

the action of CDR. Purified rat brain CDR was able to activate tryptophan 5-monooxygenase of Fraction I to the same extent as Fraction II (Table I). Furthermore, addition of both Fraction II and CDR did not activate more than either of the two alone, indicating that Fraction II and CDR may act by similar mechanisms. These results provide further evidence that the activating component in Fraction II may be identical to CDR.

The time course of activation of activator-depleted tryptophan 5-monooxygenase of Fraction I by incubation with the purified rat brain CDR is shown in Fig. 2. Incubation at 30 °C

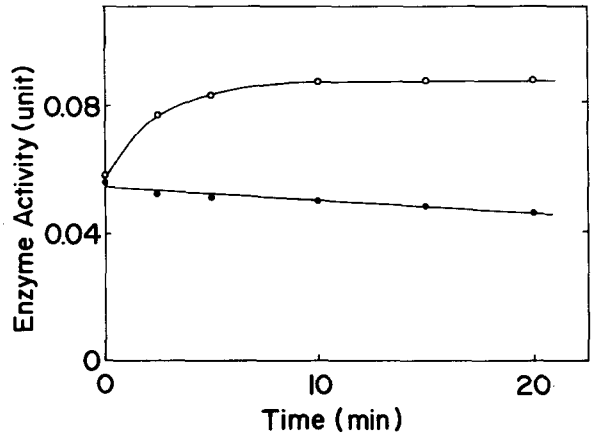

Fig. 2. Time course of activation of tryptophan 5-monooxygenase by CDR. Tryptophan 5-monooxygenase (Fraction I, 432 μg of protein) was incubated in a mixture containing 50 mM Hepes buffer, pH 7.1, 500 μM ATP, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM EGTA and 5 mM NaF in the presence (o) or absence (●) of 3.5 μg of brain CDR, in a total volume of 200 μl at 30 °C. At the indicated times, 200 μl of the assay mixture for tryptophan 5-monooxygenase containing 100 mM Na/phosphate buffer, pH 7.1, 800 μM tryptophan, 600 μM 6-MPH_4, 4 mM dithiothreitol, 5 mM EDTA and 100 μg of catalase were added and the enzyme activity was assayed as described in Experimental Procedures.

Table II. Requirements for activation of tryptophan 5-monooxygenase

System	Tryptophan 5-monooxygenase activity	
	unit	%
Complete	0.113	254
- ATP	0.046	103
- $MgCl_2$	0.043	96
- $CaCl_2$	0.045	100
- brain CDR	0.045	100

Brainstem tryptophan 5-monooxygenase (Fraction I, 432 μg of protein) was incubated under standard conditions and the enzyme activity was assayed as described under "Experimental Procedures."

resulted in a progressive increase of tryptophan 5-monooxygenase activity. The maximum activation of the enzyme was about 2-fold under the experimental conditions. The incubations were

carried out in the presence of both 100 μM EGTA and 100 μM $CaCl_2$. The concentration of free Ca^{2+} in the incubation mixture was calculated to be 3 μM or 46 μM by using an apparent binding constant for Ca^{2+}·EGTA of 7.6 X 10^6 M^{-1} (16) or 4.4 X 10^5 M^{-1} (17), respectively. As shown in Table II, the activation of tryptophan 5-monooxygenase by brain CDR required the presence of ATP, Mg^{2+} and Ca^{2+}. This activation was completely blocked by the omission of any of these. These results suggest that a CDR-dependent protein kinase may be involved in the activation of tryptophan 5-monooxygenase and that Fraction I may contain the CDR-dependent protein kinase.

The Ca^{2+}-dependent regulator protein, initially reported by Kakiuchi and co-workers (18) and by Cheung (19) as an activator protein of cyclic nucleotide phosphodiesterase, has been reported to exhibit multiple calcium-dependent regulating activities, including activation of adenylate cyclase (20,21) and activation of erythrocyte membrane (Ca^{2+}/Mg^{2+})-ATPase (22,23) as well as activation of phosphodiesterase, in a variety of tissues. More recently, it has been shown to mediate the Ca^{2+} activation of several protein kinases such as myosin light chain kinase (24,25), phosphorylase kinase (26) and glycogen synthase kinase (27). Calcium ion is known to play a number of important roles in the functioning of the nervous system, especially in the release and biosynthesis of neurotransmitters, but very little is known about biochemical events underlying the Ca^{2+}-dependent physiological process in the nervous system. The evidence described in this and the previous communication suggests that the Ca^{2+}-dependent protein kinase involved in the regulation of tryptophan 5-monooxygenase activity in rat brain may be a CDR-dependent protein kinase. Dabrowska and Hartshorne reported

that CDR mediated the Ca^{2+} activation of myosin light chain kinase from bovine brain (27). Schulman and Greengard observed CDR- and Ca^{2+}-dependent protein phosphorylation of brain membrains (28). We are currently investigating the nature of CDR-dependent protein kinase which activates tryptophan 5-monooxygenase in rat brain.

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