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DEMONSTRATION OF THE PHOSPHORYLATION-DEPENDENT INTERACTION OF TRYPTOPHAN HYDROXYLASE WITH THE 14-3-3 PROTEIN

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SUMMARY: The molecular mechanism of the phosphorylation-dependent activation of tryptophan hydroxylase is studied with respect to the role of the 14-3-3 protein. Reexamination of the system reconstituted with the purified TRH and the 14-3-3 protein showed that the level of the TRH activity correlated with the extent of the Ca^{2+}/calmodulin- or the cAMP-dependent phosphorylation in TRH. The experiment confirmed the requirement of the 14-3-3 protein for the activation, but the 14-3-3 protein added into the assay mixture did not affect either the extent nor the specificity of the phosphorylation. However, the analysis of the assay mixture on a pteridine-based affinity column indicated the formation of a complex between TRH and the 14-3-3 protein, where the complex formation depended on the phosphorylation of TRH. The complex between the phosphorylated TRH and the 14-3-3 protein could also be detected by the analysis of crude brainstem extract previously phosphorylated by endogeneous Ca^{2+}/calmodulin-dependent protein kinase. The 14-3-3 protein, therefore, appears to be a phosphorylation-dependent TRH-binding protein whose interaction causes the activation of TRH.

Tryptophan hydroxylase (TRH; EC 1.14.16.4) is the initial and rate-limiting enzyme in the biosynthesis of serotonin, and therefore the regulation of this enzyme is of physiological importance. Recent studies have revealed that the TRH activity is controlled by a variety of cellular mechanisms including enzyme induction, increased biosynthesis of cofactor tetrahydrobiopterin, and phosphorylation of the enzyme through the action of protein kinases. Among the mechanisms, phosphorylation of the enzyme is believed to be of functional significance with respect to stimulus-response coupling [1-3]. Phosphorylation of TRH by PKII

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Abbreviations used: TRH, tryptophan hydroxylase; TH, tyrosine hydroxylase; PKII, Ca^{2+}/calmodulin-dependent protein kinase type II; PKA, cAMP-dependent protein kinase; MePteH4, 2-amino-4-hydroxy-6-methyltetrahydropteridine; Me2PteH4, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, poly (vinylidene difluoride).

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or PKA causes significant enzyme activation; however, the activation requires an additional protein now identified as the family of proteins called 14-3-3 [4-6]. The 14-3-3 protein has also been shown to be essential for the PKII-dependent activation of tyrosine hydroxylase (TH; EC 1.14.16.2), the rate-limiting enzyme in the synthesis of catecholamines [7, 8].

The molecular mechanism of action of the 14-3-3 protein in these activation processes has not been established. Previous studies suggested that the 14-3-3 protein had little effect either on the kinetic properties of nonphosphorylated TRH and TH [7, 8] nor on the phosphorylation reaction itself at least in the PKII-TH system [7]. It was also shown that the 14-3-3 protein was not phosphorylated by PKII during the activation of TRH [6].

In this study we examine the role of the 14-3-3 protein in the PKII/PKA-TRH system. By using the purified preparations of TRH and the ζ_2 isoform of bovine brain 14-3-3 protein [9] we demonstrate the formation of a complex between the 14-3-3 protein and the phosphorylated TRH.

MATERIALS AND METHODS

Materials; TRH was purified from rat brainstem by pteridine affinity chromatography [4]. The 14-3-3 ζ_2 protein and PKII were purified from bovine brain [6, 10]. The catalytic subunit of PKA (56 pmol units/μg protein) was obtained from SIGMA. An antibody to the 14-3-3 protein was prepared in rabbit [11]. [γ-^{32}P] ATP (3000 Ci/mmol) and [^{125}I] anti-rabbit IgG F(ab')$_2$ (5-20 μCi/μg protein) were purchased from Amersham International.

Phosphorylation of TRH; Phosphorylation of TRH (2.8 μg) was carried out in a reaction mixture (50 mM Hepes, pH 7.6, 5 mM $Mg(CH_3COO)_2$, 0.1 mM $CaCl_2$, 0.5 mM ATP, 8 μCi of [γ-^{32}P] ATP, 1 μg of calmodulin, 1 μg of PKII, and 2 μg of ζ_2 protein) in a final volume of 150 μl. The reaction was performed at 30 °C for various time intervals (indicated in the text), and was terminated by the addition of 1 mM ATP. Proteins were analyzed by SDS/10 % (w/v) polyacrylamide gel electrophoresis followed by autoradiography. ^{32}P incorporation was measured by scintillation counting of excised TRH bands. PKII had previously been autophosphorylated with nonradioactive ATP for 10 min at 30 °C because the phosphorylated PKII gave a band close to TRH. Phosphorylation of TRH by PKA was carried out as described above except that the incubation mixture contained 50 mM Hepes (pH 7.6), 5 mM $Mg(CH_3COO)_2$, 2 mM dithiothreitol, and 0.5 μg of the catalytic subunit of PKA.

Measurement of TRH activity; TRH was phosphorylated by PKII or PKA for various time intervals (indicated in the text), and then mixed with the standard assay mixture in a final volume of 0.2 ml. The standard assay mixture contained 50 mM Hepes (pH 7.6), 0.4 mM tryptophan, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 0.3 mM $MePteH_4$, 2 mM dithiothreitol, 50 μg of catalase and 2 μg of ζ_2 protein (final concentration). The reaction proceeded for 5 min at 30 °C and was stopped by the addition of 20 μl of 60 %(w/v) perchloric acid. After the resulting precipitate was removed by centrifugation, 5-hydroxytryptophan was measured fluorometrically [7].

Binding assay ; TRH was phosphorylated for 20 min by PKII in the presence of the ζ_2 protein. The sample (0.1 ml) was applied to a pteridine-agarose column (0.1 ml bed volume) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol, 0.06 % Tween 20 and 50 μM EDTA, and incubated for 15 min at room temperature. After washing the column 4 times with 0.1 ml of 50 mM Hepes(pH 7.6), bound proteins were eluted with 0.2 ml of 10 mM $NaHCO_3$/NaOH (pH 10.8). The recovered proteins were separated by SDS/10 % (w/v) polyacrylamide gel electrophoresis, transferred to a PVDF membrane and then stained with amido black or with the 14-3-3 antibody coupled with the ^{125}I-labeled secondary antibody. For the analysis of the rat brainstem extract (25-55% saturated $(NH_4)_2SO_4$ fraction), the extract (500 μg protein) was incubated in a buffer (0.1 ml) containing 50 mM Hepes (pH 7.6), 5 mM $Mg(CH_3COO)_2$, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.5 M NaF, and 4 μg of calmodulin at 30 °C for 20 min and then analyzed as above, except that the washing buffer contained 0.2 M NaCl.

RESULTS

Fig. 1 shows the time course of the PKII/PKA-dependent phosphorylation reaction of TRH in the presence or absence of the 14-3-3 ζ2 protein. The 14-3-3 protein had no direct effect on the rate or the final level of the TRH phosphorylation. In addition, the 14-3-3 protein did not alter the site of phosphorylation in the TRH molecule because no difference was detected between the ζ2 protein plus/minus phosphopeptide maps of TRH following the digestion of the phosphorylated enzymes with lysylendopeptidase (data not shown). To examine whether the level of phosphorylation correlates with the extent of enzyme activation, TRH was first phosphorylated by PKII or PKA to various extents and the activity of the resulting enzyme was assayed in the presence of the ζ2 protein. As shown in Fig. 2, the activity levels paralleled the level of phosphorylation (compare with Figure 1). No activation occured without phosphorylation nor without the ζ2 protein (Fig. 2, dotted line), indicating that both enzyme phosphorylation and the 14-3-3 protein are necessary for the activation. It is also evident that the 14-3-3 protein could activate TRH only after the enzyme was phosphorylated. Kinetic studies revealed that this activation is due to an increase in the V_{max} value of the enzyme.

Based on these and previous results [5, 7] we assumed that the 14-3-3 protein might activate TRH through direct binding with the phosphorylated enzyme. To test this assumption a direct binding assay was performed. TRH was incubated with the ζ2 protein under phosphorylating conditions (see Methods), and the mixture was passed through an agarose column coupled with an analog of a TRH cofactor, Me2PteH4, that binds TRH. The bound proteins were eluted from the column, subjected to SDS-PAGE, and after transfer to a PVDF membrane the blot was analyzed for TRH and the 14-3-3 protein (Fig. 3a). This analysis revealed that the ζ2 protein bound to the phosphorylated TRH, as could be demonstrated by its co-elution from the column with the enzyme (lane 6). The ζ2 protein did not bind to the Me2PteH4 column under the

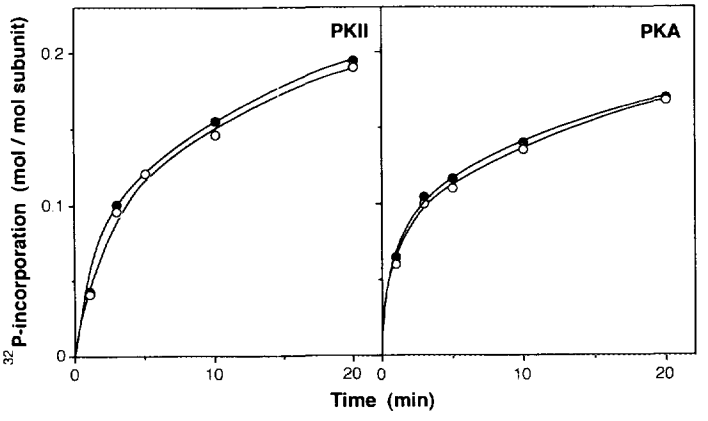

Figure 1. Time course of the phosphorylation of TRH in the presence or absence of the 14-3-3 ζ2 protein. TRH (2.8 μg) was phosphorylated by PKII (1 μg) or PKA (0.5 μg) in the presence (●) or absence (○) of the ζ2 protein. At the times indicated, the phosphorylation of TRH was measured as described under "Materials and Methods". No radioactivity was detected in the ζ2 protein under these conditions.

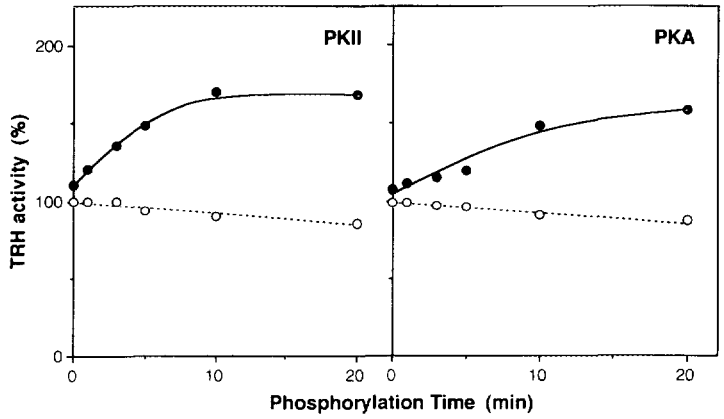

Figure 2. Relationship between phosphorylation and activation of TRH. TRH (2.8 μg) was phosphorylated by PKII (1 μg) or PKA (0.5 μg) for various time intervals, and then assayed for the activity in the presence (●) or absence (○) of the ζ2 protein. The results are expressed as a percentage of the activity in the absence of the ζ2 protein at 0 min.

nonphosphorylating condition, *i.e.* without the phosphorylation of TRH, while nonphosphorylated TRH bound to the column even without the ζ2 protein (lane 6').

In order to examine whether this complex of the phosphorylated TRH and the 14-3-3 protein could also be detected in a more crude protein mixture, we performed similar experiments using the brainstem extract. The extract was treated under the PKII-dependent phosphorylation condition (see Methods), loaded onto the Me2PteH4 column, and bound proteins were analyzed as described above. As shown in Fig. 3b (lane 3), the 14-3-3 protein was detected in a fraction co-eluted with the TRH, suggesting that binding occured in the crude extract. Again, no significant binding of the 14-3-3 protein was observed when the 14-3-3 alone or the crude extract prepared under the nonphosphorylating condition was analyzed (Fig. 3b, lanes 1 and 2). This result demonstrated the specificity of the complex formation and confirmed that the phosphorylation of the enzyme was essential for its interaction with the 14-3-3 protein.

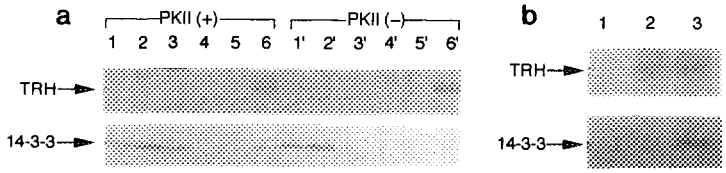

Figure 3. Binding of the 14-3-3 protein to the phosphorylated TRH. (a) TRH (2.8 μg) was phosphorylated by PKII (0.5 μg) for 20 min in the presence of the ζ2 protein (0.4 μg), and the mixture was applied to a Me2PteH4 column. After collecting the flow through (lane 1), the column was washed 4 times with 50 mM Hepes (pH 7.6) (lanes 2-5), and then bound proteins were eluted with 10 mM $NaHCO_3$/NaOH (pH 10.8) (lane 6). Proteins were subjected to SDS-PAGE, transferred to a PVDF membrane and stained with amidi black for the detection of TRH (upper panel) or with the 14-3-3 antibody for the detection of the ζ2 protein (lower panel). The control experiment was performed in the absence of PKII (lanes 1'-6'). (b) The ζ2 protein (5 μg, lane 1) or rat brainstem extract (500 μg protein, lanes 2 and 3) were incubated under nonphosphorylating (lane 2) or phosphorylating (lane 3) condition and then analyzed as above. Upper panel, amido black staining; lower panel, immunostaining with the 14-3-3 antibody.

DISCUSSION

In this study we characterized the phosphorylation-dependent activation of TRH and the role of the 14-3-3 protein. We found that the 14-3-3 protein bound with TRH in a phosphorylation-dependent manner. Since the 14-3-3 protein had no direct effect on the PKA- or PKII-dependent phosphorylation of TRH (Fig. 1), this binding appeared to be responsible for the observed protein kinase-dependent activation of the enzyme (Fig. 2). If we assume that all of the TRH and 14-3-3 molecules bound to the pteridine column (Fig. 3a, lane 6) participated in complex formation, the composition of the complex can roughly be estimated as one 14-3-3 subunit per 20 TRH monomers on the basis of the densitometric quantitation of the spots in Fig. 3a, lane 6. Because the 14-3-3 protein is dimeric [4] and TRH is a tetrameric protein [12] this corresponds to binding of one mole of 14-3-3 dimer with 10 moles of tetrameric TRH. On the other hand, TRH is phosphorylated to the level of approx. 0.2 mol per mol of TRH monomer in the assay condition employed (Fig. 1). Taking into account that the binding of the 14-3-3 protein is specific to the phosphorylated form of TRH, the binding stoichiometry can be recalculated as one mole of the 14-3-3 protein binds with 2 moles of the phosphorylated TRH. This stoichiometry was not changed when the binding experiments were performed in the presence of other additives such as tryptophan, dithiothreitol or ferrous ion, or by the use of a column packed with $MePteH_4$ instead of Me_2PteH_4. However, since our estimate is based on a semi-quantitation of the proteins stained on a gel, further refinements are probably needed to characterize the TRH/14-3-3 complex in more detail.

Recently, the 14-3-3 protein has been shown to modulate the activity of protein kinase C [9, 13]. Two sites (amino acids 60-63 and 127-142 on the ζ subunit sequence [9]) have been proposed as the sites of interaction with protein kinase C [13, 14]. We prepared the peptides including these sites by chemical synthesis (amino acids 127-142) or by limited proteolysis with lysylendopeptidase (amino acids 10-76) and examined their activity toward TRH. However, both these peptides had no effect at least toward the activity of TRH. Likewise, the treatment of ζ_2 protein with N-ethylmaleimide, a blocking agent of sulfhydryls that is purported to inhibit the 14-3-3-mediated exocytosis of catecholamines [15], did not affect the activator activity of ζ_2 protein (data not shown). Thus, the different or additional region of the 14-3-3 protein might be necessary for the activation of TRH. The studies are now in progress to identify fragment(s) which are responsible for the activation of the phosphorylated TRH.

Recent studies have indicated that protein phosphorylation regulates the cell signaling process through modification of protein-protein interaction. For instance, phosphorylation is essential for the interaction between rhodopsin and arrestin [16] and β-adrenergic receptor and β-arrestin [17]. Likewise, protein phosphorylation dissociates the protein complexes in a variety of systems including MARCKS/actin filament [18], synapsin I/synaptic vesicle-associated form of PKII [19], and GAP 43/calmodulin [20]. In each case, the alteration of the protein-protein interaction leads to biological response. In this view point, our results concerning the TRH/14-3-3 protein is an another example for the phosphorylation-dependent protein interaction that could serve for the regulation of the cellular levels of biogenic amines.

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