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Isolation of a Glycogen Synthase I Kinase That Is Independent of Adenosine 3′:5′-Monophosphate

(cyclic-AMP-dependent protein kinase/protein kinase inhibitor/casein phosphorylation/ rabbit kidney medulla)

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ABSTRACT **Three protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) were detected when the soluble fraction of rabbit kidney medulla was chromatographed on DEAE-cellulose with a linear NaCl gradient. The first two kinases eluted (Peak I and Peak II) were cyclic-AMP-dependent, whereas Peak III was cyclic-AMP-independent. A procedure was developed to separate the catalytic subunit of Peak II cyclic-AMP-dependent protein kinase (representing the bulk of the histone kinase activity) from Peak III protein kinase. In contrast to the catalytic subunit, Peak III protein kinase phosphorylated casein more rapidly than histone. Peak III was insensitive to the heat-stable protein inhibitor of cyclic-AMP-dependent protein kinases and appeared to have a higher requirement for ATP than did the catalytic subunit. Peak III catalyzed the conversion of glycogen synthase (UDPglucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11) from the I (glucose-6-phosphate-independent) to the D (glucose-6-phosphate-dependent) form. This conversion was dependent on Mg^{2+} and ATP and was unaffected by cyclic AMP, cyclic GMP, or the protein inhibitor. Glycogen synthase I in the soluble fraction of kidney medulla could be converted to the D form by endogenous glycogen synthase I kinase if Mg^{2+} and ATP were added. Most of this glycogen synthase I kinase activity was unaffected by cyclic AMP or by the protein inhibitor, suggesting that Peak III may be of major importance in the regulation of glycogen synthase *in vivo*.**

Glycogen synthase [UDPglucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11) exists in two interconvertible forms (1). Phosphorylation of glycogen synthase converts the I [glucose-6-phosphate (G-6-*P*)-independent] form to the D (G-6-*P*-dependent) form (1). In early work using relatively crude enzyme preparations from skeletal muscle, it was shown that cyclic AMP stimulated the MgATP-dependent conversion of glycogen synthase I to D (2–4). Soon after the discovery of the cyclic-AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) by Walsh *et al.* (5), it was shown by Larner and coworkers (6, 7) and by Krebs and coworkers (8) that cyclic-AMP-dependent protein kinase exhibited glycogen synthase I kinase activity. There is evidence that the cyclic-AMP-dependent protein kinase is involved in the regulation of synthase activity in many mammalian tissues (9).

The addition of cyclic AMP or certain agents to the cyclic-AMP-dependent protein kinase results in dissociation of the enzyme into catalytic subunits (which are active without cyclic AMP) and regulatory subunits (which bind cyclic AMP) (reviewed in ref. 9). We have extensively purified the catalytic subunit of cyclic-AMP-dependent protein kinase from rabbit renal medulla. In addition we have isolated another cyclic-AMP-independent protein kinase that catalyzes the MgATP-dependent conversion of synthase I to synthase D. We have also studied kidney extracts to investigate the possible physiological significance of these kinases in the regulation of glycogen metabolism.

Abbreviations: Cyclic AMP, adenosine 3′:5′-monophosphate; cyclic GMP, guanosine 3′:5′-monophosphate; G-6-*P*, glucose 6-phosphate.

MATERIALS AND METHODS

Protein Kinase Assay. Protein kinase activity was determined as described previously (10). Either casein (dephosphorylated as described in ref. 14) or histone (Type II A, Sigma) at a concentration of 6 mg/ml was used as substrate. Either 60 mM Tris·HCl containing 6 mM EDTA at pH 7.7 or 30 mM glycerophosphate at pH 7 were used as buffers. The concentration of ATP in the reaction mixture was 0.2 mM and that of magnesium acetate was 10 mM. The total reaction volume was 100 μl. The results are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to protein in 10 or 15 min/ 50 μl of reaction mix. One unit of activity is defined as the amount of enzyme that catalyzes incorporation of 1 pmol of ^{32}P per min into histone (Type II-A, Sigma Chemical Co.) for the catalytic subunit or into casein for Peak III protein kinase.

Glycogen Synthase I Kinase Assay. Synthase I kinase activity was assayed by measuring the decrease in synthase I upon incubation with MgATP (6). Synthase I essentially free of synthase I kinase activity was purified from rabbit skeletal muscle as previously described (11). A small aliquot of the synthase I was diluted to the desired activity in 25 mM glycerophosphate, 1 mM EDTA, sucrose (100 mg/ml), rabbit liver glycogen (2 mg/ml), and 12.5 mM mercaptoethanol, pH 7.0. The diluted enzyme was incubated for 20 min at 30° to fully reactivate synthase I. The synthase I kinase reaction mixture contained 0.3 units/ml of synthase I (1 unit = 1 μmol of glucose incorporated into glycogen per min at 30°), varied amounts of synthase I kinase, 25 mM glycerophosphate, 1 mM EDTA, 6 mM mercaptoethanol, sucrose (50 mg/ml), glycogen (1 mg/ml), 6 mM $MgCl_2$, and 5 mM ATP (pH 7.0). The total volume varied from 300 to 500 μl depending upon the number of time points. The reaction was initiated by the addition of MgATP and incubated at 30°. At several time points 30 μl aliquots were removed and added to 60 μl

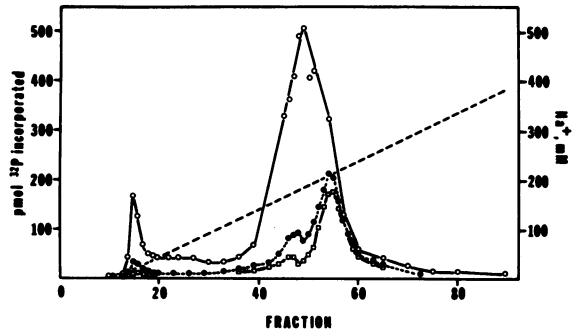

FIG. 1. DEAE-cellulose chromatography of protein kinase. Nine grams of fresh rabbit renal medulla was homogenized in 18 ml of 4 mM EDTA (pH 7.0). The homogenate was centrifuged at 38,000 × g for 30 min. The supernatant was filtered through glass wool and equilibrated with 5 mM Tris·HCl and 1 mM EDTA (pH 7.5) by Sephadex G-25 chromatography. The equilibrated supernatant was applied to a 1.5 × 26 cm DEAE-cellulose (Whatman DE-52) column which had been equilibrated in the same buffer. After a buffer wash, the protein kinases were eluted with a 400 ml linear NaCl gradient to 400 mM NaCl in the same buffer. Fractions of approximately 4 ml were collected and assayed for protein kinase activity with histone as the substrate in the presence of 2 μM cyclic AMP (O——O) and with casein as the substrate in the presence (●- - -●) or absence (□——□) of 2 μM cyclic AMP. Peaks I, II, and III refer to fractions 14–20, 42–49 and 51–60, respectively.

of synthase reaction mixture (12) containing 20 mM EDTA to stop the kinase reaction and 25 mM KF to inhibit phosphatases (6). Synthase activity was then determined by the incorporation of [^{14}C]glucose from UDP[^{14}C]glucose into glycogen as previously described (12, 13). The synthase

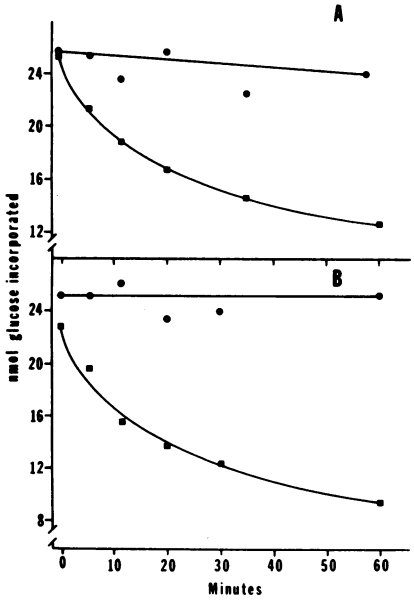

FIG. 2. Glycogen synthase I kinase activity of catalytic subunit and of Peak III protein kinase. Glycogen synthase I kinase activity was determined as described in the *text*. In A, synthase I activity was assayed after incubation with (■) or without (●) 1400 histone units/ml of catalytic subunit. In B, synthase I activity was assayed after incubation with (■) or without (●) 2800 casein units of Peak III protein kinase. There was no significant change in total synthase activity (data not shown).

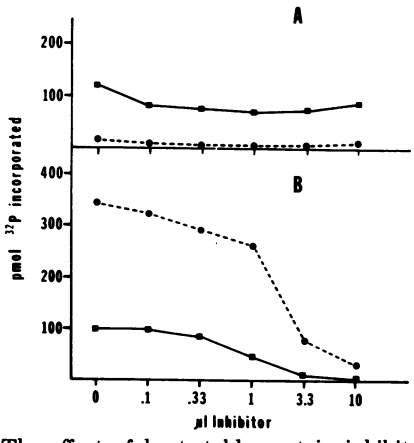

FIG. 3. The effect of heat-stable protein inhibitor on Peak III protein kinase and on the catalytic subunit. Protein kinase assays were conducted at pH 7 as described in the text with various amounts of heat-stable protein inhibitor. In A, Peak III protein kinase was assayed and in B catalytic subunit was assayed with casein (■——■) or histone (●- - -●) as substrate. The heat-stable protein inhibitor was prepared through the 15% trichloroacetic-acid step (17). The protein concentration of the inhibitor solution as determined by a modified Lowry method (18) was 18.2 mg/ml.

activity ratio is defined as the activity in the presence of 10 mM Na_2SO_4 (synthase I) divided by the activity in the presence of 6.7 mM glucose 6-phosphate (total synthase).

RESULTS

DEAE-Cellulose Chromatography of Protein Kinases. When a kidney extract was fractionated on DEAE-cellulose, three peaks of casein kinase activity were detected. These were designated as Peak I, Peak II, and Peak III (Fig. 1). When histone was used as substrate only two peaks were resolved. These corresponded approximately to Peak I and Peak II. Peak I and Peak II phosphorylated histone more rapidly than casein and were stimulated by cyclic AMP. Judged by the salt required for elution and on the ease of dissociation into subunits (unpublished), Peak I and Peak II are similar to the two forms of cyclic-AMP-dependent protein kinase previously described in skeletal muscle (14) and in other tissues (15). Peak III, on the other hand, was not stimulated by cyclic AMP and phosphorylated casein more rapidly than it did histone.

Purification of Protein Kinases. The major cyclic-AMP-dependent protein kinase (Peak II) and the cyclic-AMP-independent protein kinase (Peak III) were only partially resolved by DEAE-cellulose chromatography. Therefore, a procedure was developed to separate these two kinases. One hundred and fifty grams of frozen rabbit kidney medulla (Pel-Freeze, Rogers, Arkansas) was homogenized in 300 ml of 10 mM potassium phosphate containing 0.1 mM dithiothreitol and 1 mM EDTA at pH 6.8. This and all subsequent operations were carried out at 4°. The homogenate was centrifuged at 10,000 × g for 30 min and the supernatant was applied to a DEAE-cellulose column (2.6 × 30 cm). The column was washed with 50 mM potassium phosphate containing 0.1 mM dithiothreitol and 1 mM EDTA at pH 6.8. Peak I, which was eluted by these conditions, was discarded. The column was then washed with the same buffer containing 10 μM cyclic AMP to elute the catalytic subunit of Peak II

TABLE 1. *Factors affecting the glycogen synthase I activity of Peak III protein kinase and of the catalytic subunit*

Enzyme	Condition	Kinase incubation (min)	Synthase activity (nmol/10 min per 75 μl) Without G-6-P	With G-6-P	Synthase activity ratio
		Experiment 1			
Peak III	Complete	0	26.6	27.5	0.97
	Complete	20	14.4	26.9	0.54
	Complete + inhibitor*	20	14.4	26.4	0.55
	0.1 mM ATP†	20	23.4	26.5	0.88
		Experiment 2			
Catalytic subunit	Complete	0	28.4	27.8	1.02
	Complete	20	14.7	24.6	0.60
	Complete + inhibitor*	20	27.0	27.4	0.99
	0.1 mM ATP†	20	15.1	25.0	0.60
		Experiment 3			
Peak III	Complete	0	18.3	19.6	0.93
	Complete	20	12.4	19.9	0.62
	Complete + 5 μM cyclic AMP	20	12.8	20.3	0.63
	Complete + 5 μM cyclic GMP	20	12.6	20.5	0.61

Synthase I kinase activity was determined as described in the *text*. Kinase activity was linear with time for 20 min in all cases. The 0 times were essentially identical for all conditions so the average is given. Peak III protein kinase was included at 3000 casein units/ml and catalytic subunit at 1500 histone units/ml. The complete system as described in the *text* contained 5 mM ATP and 6 mM $MgCl_2$.

* The inhibitor (refer to Fig. 3) was included at a concentration of 6 mg/ml.

† Same as complete system, except ATP was 0.1 mM.

cyclic-AMP-dependent protein kinase (16). The catalytic subunit was purified further by chromatography on hydroxylapatite. Details of this method will be published elsewhere. Peak III protein kinase was then eluted from the DEAE column with a linear gradient of NaCl to 0.5 M. The fractions containing enzyme were pooled and the enzyme was precipitated by the addition of 0.35 g of crystalline ammonium sulfate per ml of solution (55% saturation). The precipitate was collected by centrifugation at 10,000 × *g* for 25 min, dissolved in 25 mM glycerophosphate buffer containing 1 mM EDTA at pH 7.0, dialyzed against the same buffer, and centrifuged to remove insoluble proteins. The catalytic subunit was purified approximately 2500-fold to a specific activity of 500 histone units/μg. Peak III was purified about 24-fold to a specific activity of 1.3 casein units/μg.

Glycogen Synthase I Kinase Activity of Peak III Protein Kinase and of the Catalytic Subunit. Both Peak III protein kinase and catalytic subunit catalyzed the inactivation of glycogen synthase I without changing total synthase activity (Fig. 2). The reaction was reversed by the addition of a partially purified phosphatase (not shown). Even with high kinase levels or longer incubations, the synthase activity ratio did not drop below 0.35. Addition of both kinases simultaneously or sequentially did not alter the final synthase activity ratio.

Effect of Protein Inhibitor on Peak III Protein Kinase and Catalytic Subunit. Walsh and coworkers (17) have purified a heat-stable protein from skeletal muscle which inhibits cyclic-AMP-dependent protein kinases from a number of tissues. This inhibitor has been proposed as a useful tool for distinguishing between cyclic-AMP-independent protein kinases and the catalytic subunit of cyclic-AMP-dependent protein kinases (20). To further characterize the Peak III kinase, we determined its susceptibility to this heat-stable protein kinase inhibitor. The protein inhibitor had little effect on the activity of Peak III protein kinase when casein or histone was used as a substrate (Fig. 3A). As shown in Fig. 3B, the protein inhibitor is a very effective inhibitor of the catalytic subunit of Peak II kinase. The effect of the protein inhibitor on synthase I kinase activity of the two kinases is shown in Table 1. Synthase I kinase activity of the catalytic subunit was completely inhibited by the protein inhibitor. The same concentration of inhibitor was without effect on the synthase I kinase activity of Peak III protein kinase.

Some other properties of the synthase I kinase activity of Peak III protein kinase were investigated. The activity was dependent upon both ATP and Mg^{2+} and it was not inhibited by 4 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), suggesting that the enzyme does not require calcium. As shown in Table 1, the catalytic subunit was equally effective at 0.1 mM or 5 mM ATP while Peak III protein kinase had only partial activity at the lower ATP concentration. Neither cyclic AMP nor cyclic GMP (5 μM) had any effect on synthase I kinase activity of Peak III protein kinase (Table 1).

Glycogen Synthase I Kinase Activity in Renal Medulla Supernatant. In an attempt to determine the relative importance of cyclic-AMP-dependent and cyclic-AMP-independent synthase I kinase, we studied the kinases of the 20,000 × *g* supernatant (Fig. 4). The supernatant was assayed for histone and casein kinase activity. When histone was used as a substrate, the cyclic AMP stimulation was 7-fold. Histone kinase

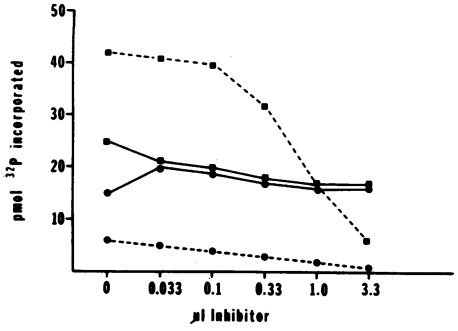

FIG. 4. Effect of heat-stable protein inhibitor on histone and casein kinase activity of the supernatant fraction from renal medulla. Fresh rabbit renal medulla (3.4 g) was homogenized in 6.8 ml of 50 mM Tris·HCl and 5 mM EDTA, pH 7.7 (Tris–EDTA). The homogenate was centrifuged at 20,000 × *g* for 20 min. The supernatant was filtered through glass wool and incubated at 30° to convert all of the synthase to the I form (19). The solution was then chromatographed on Sephadex G-25 equilibrated with Tris–EDTA. The protein peak was collected and an aliquot was diluted 11-fold with Tris–EDTA. Protein kinase assays were conducted at pH 7.7 as described in the *text*. Twenty μl of the diluted supernatant fraction and the indicated volume of heat-stable protein inhibitor were added to a final reaction volume of 100 μl. The inhibitor (refer to Fig. 3) was dialyzed for several hours against Tris–EDTA before use. Assays were performed with histone in the presence (■- - -■) or absence (●- - -●) of 2 μM cyclic AMP and casein in the presence (■——■) or absence (●——●) of 2 μM cyclic AMP.

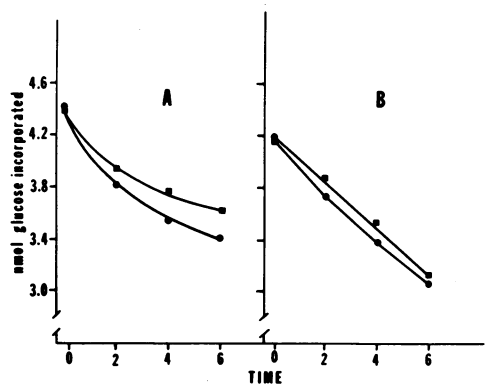

FIG. 5. Effect of cyclic AMP and heat-stable protein inhibitor on the glycogen synthase I kinase activity of the supernatant from renal medulla. The protein peak from the Sephadex G-25 column described in Fig. 4 was used as the source of both synthase I kinase and synthase I substrate. The protein peak was diluted 2-fold in the synthase I kinase assay. The reaction was initiated by addition of ATP and $MgCl_2$ to concentrations of 5 mM and 10 mM, respectively. Synthase I kinase activity was determined in the presence (●) and absence (■) of 50 μM cyclic AMP. In A, there was no inhibitor present. In B, the protein inhibitor was included at 8 mg/ml. Time is in min.

activity both in the presence and absence of cyclic AMP was inhibited by the protein inhibitor. Casein kinase activity of the supernatant was not markedly stimulated by cyclic AMP and it was for the most part insensitive to the heat-stable protein inhibitor. In Fig. 5, the synthase I kinase activity of the supernatant on endogenous synthase I is illustrated. The synthase I kinase activity was stimulated only slightly by cyclic AMP. When the heat-stable protein inhibitor was included at a concentration that inhibited cyclic-AMP-dependent histone phosphorylation by over 75%, the inhibitor had very little effect on synthase I kinase activity.

DISCUSSION

Since the discovery that glycogen synthase I was inactivated by cyclic-AMP-dependent protein kinase (6–8), numerous studies have suggested that cyclic-AMP-dependent protein kinase is the *in vivo* synthase I kinase (reviewed in ref. 9). We have now isolated from rabbit renal medulla a cyclic-AMP-independent synthase I kinase (Peak III protein kinase). This enzyme differs from the catalytic subunit of cyclic-AMP-dependent protein kinase with regard to behavior on DEAE-cellulose, protein substrate specificity, sensitivity to the heat-stable protein inhibitor, and apparent requirement for ATP. Thus, it is clear that this cyclic-AMP-independent protein kinase (Peak III protein kinase) is a different enzyme than cyclic-AMP-dependent protein kinase or its catalytic subunit.

Villar-Palasi *et al.* (7) have reported that, during the course of purification of skeletal muscle synthase I kinases, they have obtained kinase preparations that were not stimulated by cyclic AMP. In addition, they have observed a conversion of cyclic-AMP-dependent to cyclic-AMP-independent synthase I kinase. Although we have been able to convert the cyclic-AMP-dependent protein kinase to an independent form, i.e., the catalytic subunit, we have not seen any evidence for conversion of cyclic-AMP-dependent protein kinase to Peak III protein kinase. Abou-Issa *et al.* (21) recently reported on a cyclic-AMP-independent protein kinase from swine kidney which catalyzes the conversion of glycogen synthase I to the D form. However, it seems unlikely that it is identical to Peak III protein kinase, since it has a substrate specificity similar to that of the catalytic subunit of cyclic-AMP-dependent protein kinases and is tightly bound to particulate material. Nimmo and Cohen (24) have reported on a cyclic-AMP-independent kinase that phosphorylates glycogen synthase. However, this kinase did not catalyze the conversion of glycogen synthase I to the D form and therefore differs from Peak III.

We have previously suggested that a kinase other than the cyclic-AMP-dependent protein kinase may be involved in the inactivation of renal synthase I (19). This suggestion was based on the fact that cyclic AMP produced only a slight stimulation of synthase I kinase activity of whole rat kidney (22) and rabbit renal cortex and medulla (19). In the same tissues, histone phosphorylation was markedly stimulated by cyclic AMP. Furthermore, synthase I kinase activity was 3-fold greater in the cortex than in the medulla, while cyclic AMP-dependent histone kinase activity was the same in both cortex and medulla. In the present study we used the protein inhibitor to assess the relative contribution of cyclic-AMP dependent and cyclic-AMP-independent synthase I kinase in supernatants obtained from renal medulla. Most of the synthase I kinase activity was cyclic-AMP-independent and insensitive to the protein inhibitor. Thus, cyclic-AMP-independent protein kinase may play an important role in the regulation of glycogen metabolism in this tissue. Since cyclic-AMP-independent protein kinases have been reported in a wide variety of tissues (reviewed in ref. 23), it is further possible that the enzyme described here has wide-spread importance in the regulation of glycogen synthesis.

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