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Stephen J. Beebe
Old Dominion University, sbeebe@odu.edu

Erwin M. Reimann

Keith K. Schlender

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Purification and Characterization of a cAMP- and Ca^{2+}-Calmodulin-independent Glycogen Synthase Kinase from Porcine Renal Cortex*

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Stephen J. Beebe‡§, Erwin M. Reimann¶‖, and Keith K. Schlender‡**

From the Departments of ‡Pharmacology, and ¶Biochemistry, Medical College of Ohio, Toledo, Ohio 43699

We recently reported the partial purification of a cAMP-independent and Ca^{2+}-calmodulin-independent glycogen synthase kinase from porcine renal cortex (Schlender, K. K., Beebe, S. J., and Reimann, E. M. (1981) *Cold Spring Harbor Conf. Cell Proliferation*, 389–400). Subsequent purification indicated that the enzyme preparation consisted of at least three forms of glycogen synthase kinase which could be resolved by ATP gradient elution from aminoethylphosphate-agarose (AEP-agarose). The predominant form of glycogen synthase kinase, which eluted from AEP-agarose between 2 and 6 mM ATP, was purified approximately 800-fold and is designated GSK-A1. It had a molecular weight of 45,000–50,000 as determined by gel filtration and sucrose density gradient centrifugation. It catalyzed the transfer of 1 mol of ^{32}P/mol of synthase subunit into a low molecular weight (10,000) CNBr peptide which was tentatively identified as Ser-7 (site 2) by high performance liquid chromatography. This phosphorylation decreased the activity ratio (activity in the absence of glucose-6-P divided by activity in the presence of 7.2 mM glucose-6-P) from 0.95 to about 0.55. GSK-A1 appeared to be specific for and had low $s_{0.5}$ values for both substrates, ATP (13 μM) and glycogen synthase (0.3–0.4 μM). The enzyme could not use GTP as the phosphate donor. GSK-A1 was not affected by the protein kinase inhibitor, cAMP, cGMP, Ca^{2+}-calmodulin, EGTA, or trifluoperazine and had a broad pH optimum (pH 7.0–8.5). A second form, GSK-A2, was eluted from AEP-agarose between 7 and 9 mM ATP. GSK-A2 could transfer a 2nd mol of ^{32}P/mol of synthase subunit and decreased the activity ratio to 0.30. The interrelation among these multiple forms is not clear, but the data suggest that multiple kinases are required to form the highly inactivated glycogen synthase in renal tissues.

Glycogen synthase is the rate-limiting enzyme in glycogen synthesis (1). The complex control of the enzyme involves allosteric regulation as well as covalent modification via phosphorylation-dephosphorylation which is under hormonal control (2–4). Picton *et al.* (5) have identified seven phosphorylation sites on synthase. They have designated the sites as 1a, 1b, 2, 3a, 3b, 3c, and 5. They have further shown that site 2 is Ser-7 (5). As phosphate is added to the various sites, less active forms of synthase are produced with different kinetic parameters for the allosteric activator glucose-6-P and the substrate UDPglucose (6).

It is generally accepted that several well characterized kinases can phosphorylate and inactivate glycogen synthase. These include skeletal muscle cAMP-dependent protein kinase (7, 8) which is regulated by intracellular levels of cAMP and skeletal muscle phosphorylase kinase (9–12) which is regulated by Ca^{2+} and calmodulin (13, 14). Payne and Soderling (15) and Ahmad *et al.* (16) have shown that liver contains a Ca^{2+}-calmodulin-dependent kinase that is distinct from phosphorylase kinase, and Woodgett *et al.* (17) have isolated a similar kinase from skeletal muscle. Recently, it has become clear that a phosvitin/casein kinase is capable of catalyzing phosphorylation but not inactivation of glycogen synthase. This kinase has been designated $PC_{0.7}$ by DePaoli-Roach *et al.* (18, 19), casein kinase 2 by Huang *et al.* (20), and GSK-5 by Cohen *et al.* (21). In addition, glycogen synthase is phosphorylated by other cyclic nucleotide-independent and Ca^{2+}-calmodulin-independent glycogen synthase kinases whose regulatory mechanisms have not yet been elucidated (18, 21, 22).

Most of the work with glycogen synthase kinase has been done on enzymes purified from skeletal muscle. However, Schlender and Reimann (22) showed that among several rat tissues, kidney was the most abundant source of cAMP-independent glycogen synthase kinase. Little is known about the regulation of glycogen synthase in tissues such as the kidney with low concentrations of glycogen. The demonstration that the kidney cortical glycogen synthase activity ratio was low and the $A_{0.5}$ for glucose-6-P was high suggested that the synthase is highly phosphorylated (23). Given the predominance of cAMP-independent glycogen synthase kinase in this tissue (24) and the absence of Ca^{2+}-calmodulin-stimulated glycogen synthase kinase activity (23), it is highly likely that the cAMP- and Ca^{2+}-calmodulin-independent glycogen synthase kinases are responsible for these properties of renal cortical glycogen synthase. We recently reported on the partial purification of GSK-P[1] from porcine renal cortex (23).

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§ Current address, Howard Hughes Medical Institute, Department of Physiology, Vanderbilt University, Nashville, TN 37232.

‖ Recipient of National Institutes of Health Career Development Award AM 00446.

** Recipient of National Institutes of Health Career Development Award AM 00337. To whom correspondence should be addressed.

[1] The abbreviations used are: GSK-P, forms of glycogen synthase kinase which bind to phosphocellulose and elute at 0.5 M NaCl; GSK-D, forms of glycogen synthase kinase which bind to DEAE-cellulose but do not bind to phosphocellulose; AEP-agarose, aminoethylphosphate-agarose; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; GSK-A1, form of glycogen synthase kinase eluted from AEP-agarose by 2–6 mM ATP; GSK-A2, form of glycogen synthase kinase eluted from AEP-agarose by 7–9 mM ATP; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MES, 4-morpholinethanesulfonic acid.

We now report that a recently developed agarose resin containing an aminoethylphosphate group can be used to isolate multiple forms of glycogen synthase kinase which are present in porcine renal cortex. At least three forms of glycogen synthase kinase were separated by ATP gradient elution of glycogen synthase kinase from AEP-agarose. The major form of glycogen synthase designated GSK-A1 was purified and characterized.

EXPERIMENTAL PROCEDURES

Materials—Rabbit skeletal muscle glycogen synthase was prepared through the polyethylene glycol precipitation step followed by phosphocellulose chromatography (glycogen-free) or through the ethanol precipitation step (glycogen-bound) as previously described (25). The inhibitor of the cAMP-dependent protein kinase (26) and catalytic subunit of cyclic AMP-dependent protein kinase (27) were prepared as previously described. Phosphorylase *b* was prepared from rabbit skeletal muscle by the method of Fischer and Krebs (28). Phosphorylase kinase was prepared from rabbit skeletal muscle through the Sepharose 4B gel filtration step (29) or was a gift from Dr. Ted Chrisman (Howard Hughes Medical Institute, Department of Physiology, Vanderbilt School of Medicine). [γ-^{32}P]ATP was prepared according to the method of Walseth and Johnson (30). Dr. Simon Pilkis (Department of Physiology, Vanderbilt School of Medicine) kindly provided the following: 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase, and fructose 2,6-bisphosphate. Dr. Jackie Corbin (Howard Hughes Medical Institute, and Department of Physiology, Vanderbilt School of Medicine) generously provided beef lung cGMP-dependent protein kinase, rabbit skeletal muscle type I, and beef heart type II regulatory subunit of cyclic AMP-dependent protein kinase. Immersible Cx ultrafilters were purchased from the Millipore Corp. AEP-agarose was a product from Pierce Chemical Co. All other chemicals and reagents were obtained from sources previously indicated (25).

Swine kidneys were obtained from the local slaughterhouse. Approximately 15–30 min after the animals were killed, the kidneys were placed on ice and transported to the laboratory for dissection to remove the cortex. In some experiments fresh cortex was used and in others the tissue was stored at −70 °C for up to 4 weeks before isolation of glycogen synthase kinase. When the cortex was stored at −70 °C for longer periods of time, the recovery of glycogen synthase kinase in the first and/or second chromatography steps was significantly reduced.

Glycogen Synthase Kinase Assays—Unless otherwise indicated, glycogen synthase kinase activity was determined by measuring the inactivation of rabbit skeletal muscle glycogen synthase as previously described (25) with the following changes. The buffer was 35 mM Tris-HCl (pH 7.8 at 25 °C), and the reaction mixture contained 1 mM ATP, 2 mM $Mg(Ac)_2$, 0 or 4 mM NaCl, and 0 or 0.002% Brij 35. An inactivation unit of glycogen synthase kinase activity is defined as the amount of enzyme which causes the glycogen synthase activity measured in the absence of glucose-6-P (31) to decrease by 1 milliunit/min at 30 °C. Phosphorylation by kinases did not change the activity measured in the presence of glucose-6-P. Kinase activity was linear with time and dilution over a very narrow range, but resonable estimates of activity could be obtained when the activity ratio (activity in the absence of glucose-6-P divided by activity in the presence of 7.2 mM glucose-6-P) was reduced from the initial value of 1.0 to between 0.95 and 0.75.

Phosphorylation and inactivation of glycogen synthase was carried out as previously described (25) with the following exceptions. The reaction mixture included 50 mM Tris-HCl (pH 7.8 at 25 °C), 1.9 or 9 mM β-glycerol phosphate, and 0 or 4 mg of glycogen/ml. At various time points, aliquots were removed and analyzed for protein-bound ^{32}P or activity ratio. One glycogen synthase kinase phosphorylation unit is equal to 1 pmol of ^{32}P incorporated into glycogen synthase/min.

Purification of Glycogen Synthase Kinase from Porcine Renal Cortex—A procedure was developed to purify the major glycogen synthase kinase from porcine renal cortex. Frozen cortex (250 g) was thawed for 15 min in cold running tap water and then homogenized in a Waring blender with 625 ml of 8 mM K_2HPO_4, 2 mM KH_2PO_4, 1 mM EDTA, 0.5 mM PMSF, and 250 mM sucrose (pH 7.8). All operations were carried out at 0–4 °C unless otherwise stated. The homogenate was centrifuged for 50 min at 30,000 × *g*. The supernatant was filtered through glass wool. The clear extract was added to 190 ml of phosphocellulose which had been equilibrated in homogenizing buffer. After 1 h the suspension was filtered on a funnel equipped with a porous polyethylene plate, washed with 1 liter of homogenizing buffer, and then washed with 1 liter of homogenizing buffer (containing 150 mM NaCl) without sucrose. The resin was then suspended in the latter buffer, poured into a 5-cm diameter column, and washed with an additional bed volume of buffer containing 150 mM NaCl. The GSK-P was eluted with buffer containing 8 mM K_2HPO_4, 2 mM KH_2PO_4, 1 mM EDTA, 0.5 mM PMSF, and 500 mM NaCl (pH 7.3). In a series of nine batch adsorptions carried out in this manner, recoveries of GSK-P in the 500 mM NaCl eluates were 42.2 ± 3.3% (mean ± S.E.) and the fold purification was 16.5 ± 3 (mean ± S.E.). No additional activity was detected by the glycogen synthase kinase assay when the column was washed with 1 M NaCl, but activity could be detected by measuring ^{32}P incorporation into glycogen synthase.

The phosphocellulose eluate (GSK-P) was diluted 3-fold with 5 mM K_2HPO_4, 5 mM KH_2PO_4, 1 mM EDTA, 0.5 mM PMSF (pH 6.9), and applied to a hydroxyapatite column (2.6 × 11.3 cm) equilibrated in the above buffer containing 150 mM NaCl. After the sample was applied, the column was washed with the following sequence of buffers: 1) 3 bed volumes of 6 mM K_2HPO_4, 4 mM KH_2PO_4, 1 mM EDTA, 0.5 mM PMSF (Buffer A, pH 7.1) containing in addition 500 mM NaCl; 2) 2 bed volumes of Buffer A; and 3) 3 bed volumes of Buffer A containing 15 mM potassium citrate. These washes eluted little or no glycogen synthase kinase activity. Glycogen synthase kinase was then eluted with Buffer A containing 15 mM potassium citrate and 350 mM NaCl.

The hydroxyapatite eluate (15.5 ml) was then adjusted to pH 7.8 at 1 °C with 45 ml of 10 mM K_2HPO_4, 1 mM EDTA, and 0.01% Brij 35. The preparation was then adjusted to a final volume of 230 ml with 8 mM K_2HPO_4, 2 mM KH_2PO_4, 1 mM EDTA, 0.01% Brij 35 (pH 7.8). This diluted the NaCl in the hydroxyapatite eluate to about 20 mM. The inclusion of the detergent Brij 35 aided in the stability of glycogen synthase kinase under these conditions of high dilution. Dialysis to remove salt often resulted in greater losses of activity than when the preparation was diluted in the presence of Brij 35. The entire volume was applied to an AEP-agarose column (0.9 × 12 cm) equilibrated in 8 mM K_2HPO_4, 2 mM KH_2PO_4, and 0.01% Brij 35 (pH 7.8). After the sample had been applied, the column was washed with 2 bed volumes of equilibrating buffer. The column was eluted with a linear gradient of 0–15 mM ATP in equilibrating buffer adjusted to pH 7.8 (total volume 60 ml). The AEP-agarose column resolved glycogen synthase kinase activity into two components designated GSK-A1, eluting between 2 and 6 mM ATP, and GSK-A2, eluting between 7 and 9 mM ATP (Fig. 1*A*). Fractions were pooled as indicated in Fig. 1*A* and concentrated using an immersible Millipore filter. No additional activity could be eluted by washing the column with buffer containing 500 mM NaCl.

In addition to the forms of glycogen synthase kinase that were bound (Fig. 1*A*), about 10% of the glycogen synthase kinase activity was not bound to the column under these conditions. When the flow-through fractions from the first AEP-agarose column were rechromatographed on a second AEP-agarose column, all the glycogen synthase kinase was bound to the column (Fig. 1*B*). When the column was eluted with an ATP gradient, at least three peaks were resolved. Two small peaks of activity eluted below 2 mM ATP, and a third major peak of activity eluted between 2 and 6 mM ATP. The latter presumably represents GSK-A1 which was overloaded on the primary column.

The pooled and concentrated GSK-A1 and GSK-A2 were separately chromatographed on a Sephadex G-150 fine column (1.5 × 86 cm) (Fig. 2). Both of the glycogen synthase kinase preparations eluted as single peaks with apparent molecular weights of about 45,000. A protein peak preceded the glycogen synthase kinase activity of GSK-A1 (Fig. 2*A*). When individual fractions were separately subjected to SDS-PAGE, the protein peak which preceded the glycogen synthase kinase activity appeared as a band near 30,000 daltons (not shown). To minimize contaminating protein, only fractions 48–54 were pooled. The pool was concentrated 6-fold using the immersible Millipore filter. On SDS-PAGE this preparation still contained the 30,000-dalton protein in addition to a prominent band at 45,000 and three minor bands between 30,000 and 45,000. Fractions 42–51 from the second Sephadex G-150 column were pooled for GSK-A2 (Fig. 2*B*). The pooled fractions were concentrated 8.5-fold with the immersible Millipore filter. A summary of the purification is presented in Table I.

Gel Filtration—Sephadex G-100 or G-150 columns were calibrated

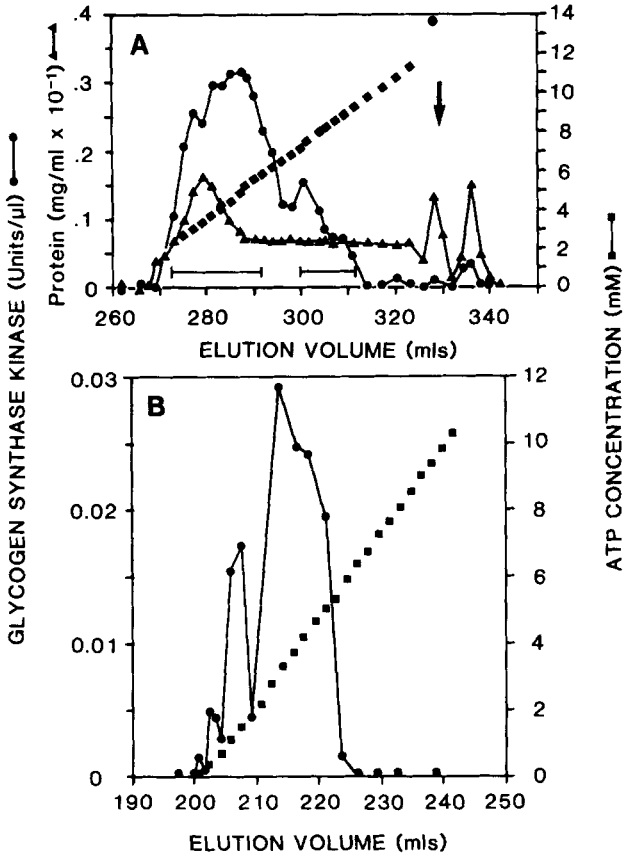

FIG. 1. **Aminoethylphosphate-agarose chromatography.** *A*, chromatography was carried out as described in the text. The proteins were eluted with a linear ATP gradient. At an elution volume of 330 ml, the column was washed with 8 mM K_2HPO_4, 1 mM EDTA, 0.01% Brij 35, 500 mM NaCl. *B*, rechromatography of AEP-agarose flow-through fractions. The flow-through fractions were pooled and rechromatographed on the same AEP-agarose column (1.6 × 2.5 cm). The proteins were eluted with a 60-ml linear gradient between 0 and 12 mM ATP. Fractions (0.85 ml) were assayed for glycogen synthase kinase activity (●), ATP (■), and protein (▲).

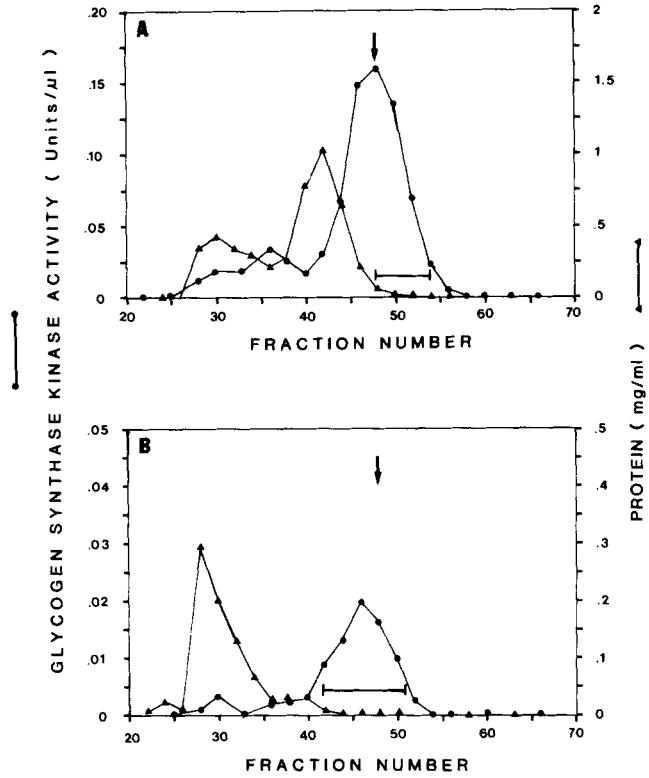

FIG. 2. **Sephadex G-150 gel filtration of GSK-A1 and GSK-A2.** *A*, concentrated GSK-A1 from an AEP-agarose column was applied to a Sephadex G-150 column equilibrated in 6 mM K_2HPO_4, 4 mM KH_2PO_4, 1 mM EDTA, 200 mM NaCl, and 0.01% Brij 35. Fractions between 48 and 54 were pooled and concentrated by Millipore filtration. *B*, concentrated GSK-A2 from an AEP-agarose column was applied to a Sephadex G-150 column as described above. Fractions between 42 and 51 were pooled and concentrated by Millipore filtration. Fractions were assayed for glycogen synthase kinase activity (●) and protein (▲). The *arrows* indicate the elution volume of ovalbumin.

using standard proteins. The proteins, their molecular weights, and their Stokes radii in nanometers were: blue dextran (void volume marker); bovine serum albumin (67,000, 3.6); ovalbumin (45,000, 2.7); catalytic subunit of cAMP-dependent protein kinase (40,000, 2.7); carbonic anhydrase (30,000, 2.3); chymotrypsinogen A (25,000, 2.4), and adenosine (inclusion volume marker).

Sucrose Density Gradient Centrifugation—Sucrose gradients were done by modification of the method described by Brandt *et al.* (32). Glycogen synthase kinase was applied to the top of a 5–25% sucrose gradient in 6 mM K_2HPO_4, 4 mM KH_2PO_4, and 1 mM EDTA (pH 7.1). Catalase (10 mg/ml, $s_{20,w}$ = 11.1) was included as an internal standard, and catalase and lactoperoxidase (10 mg/ml, $s_{20,w}$ = 5.4) were applied to a separate gradient as external standards. Centrifugation was for 16 h at 40,000 rpm in an SW 50.1 rotor in a Beckman L2-65B ultracentrifuge at 4 °C. The molecular weight and frictional ratio were calculated as described by Siegel and Monty (33).

Polyacrylamide Gel Electrophoresis—Ten or 7.5% slab SDS-PAGE was carried out as described by Laemmli (34). Gels were stained with 0.12% Coomassie brilliant blue A-250 in 7.5% acetic acid and 50% (v/v) methanol.

High Performance Liquid Chromatography—Reverse phase high performance liquid chromatography of tryptic peptides derived from phosphorylated glycogen synthase was done in collaboration with Dr. Thomas R. Soderling (Howard Hughes Medical Institute, Department of Physiology, Vanderbilt School of Medicine). This procedure will separate phosphorylation sites 1a, 1b, 2, 3abc, and 5 (35).

Other Methods—Proteins were determined by the Lowry method as modified by Hartree (36), by absorbance at 280 nm, or by the method of Bradford (37). Kinetic parameters were calculated by nonlinear regression analysis (38).

TABLE I

Purification of porcine renal cortical glycogen synthase kinase

Details of the purification procedure were as described in the text. Assay of glycogen synthase kinase was by inactivation of glycogen-bound synthase. Proteins were determined by the method of Hartree (36). AEP-agarose 1 and AEP-agarose 2 represent AEP-agarose pooled volumes 272–292 and 300–311, respectively, as indicated in Fig. 1*A*.

	Protein	Total units	Specific activity	Recovery	Purification
	mg			%	*-fold*
Extract	12,615	325,860	25.8	100	1
Phosphocellulose	395	126,620	321	39	12.4
Hydroxyapatite	28.9	51,130	1,768	16	68.5
AEP-agarose 1 (concentrated)	11.5	8,970	780	2.8	30.2
Sephadex G-150 1 (concentrated) (GSK-A1)	0.16	3,213	19,631	1.0	761
AEP-agarose 2 (concentrated)	2.8	1,000	355	0.3	13.8
Sephadex G-150 2 (concentrated) (GSK-A2)	0.16	772	4,787	0.2	186

RESULTS

Multiple Forms of Glycogen Synthase Kinase from Porcine Renal Cortex—Gel filtration of crude extracts, prepared as

described above, on Sephadex G-100 revealed several molecular weight forms of glycogen synthase kinase with activity insensitive to cAMP, Ca^{2+}-calmodulin, or the heat-stable inhibitor of cAMP-dependent protein kinase (data not shown). Compiling data from several experiments demonstrated a major glycogen synthase kinase activity peak eluting with an apparent molecular weight of 45,000–50,000. In addition there was a prominent shoulder of activity eluting at 50,000–55,000. A small peak of activity eluted at >70,000, and in some experiments activity eluted with an apparent molecular weight of 35,000. The appearance of multiple peaks of glycogen synthase kinase activity on Sephadex G-100 was unaffected by the presence of protease inhibitors (PMSF, benzamidine, and EDTA). Similar gel filtration profiles were obtained under a variety of conditions including the use of fresh or frozen tissue, homogenization with or without sucrose present, or when 50 mM Tris, 200 mM NaCl (pH 7.5), or 10 mM potassium phosphate, 200 mM NaCl (pH 7.2), or 100 mM phosphate (pH 7.2) buffer systems were used. When gel filtration column fractions were assayed for inhibitor-sensitive histone kinase activity, nearly all of the activity was cAMP-dependent and appeared in the void volume, indicating that the cAMP-dependent protein kinase chromatographed as the holoenzyme with little, if any, free catalytic subunit present.

Previous studies had shown that glycogen synthase kinase of rabbit kidney extracts could be separated into two types (GSK-D and GSK-P) by chromatography on phosphocellulose and DEAE-cellulose (24). As measured by the synthase inactivation assay, about 25% of the glycogen synthase kinase activity in swine renal cortex was GSK-D and 75% was GSK-P. GSK-D and GSK-P eluted from Sephadex G-100 with apparent molecular weights of 50,000–55,000 and 45,000–50,000, respectively. Using the synthase phosphorylation assay, a small amount of a third type of glycogen synthase kinase could be detected. The latter, which was eluted from phosphocellulose by about 1 M NaCl, also contained heparin-sensitive casein kinase activity and is likely similar to the enzyme purified from rabbit skeletal muscle designated $PC_{0.7}$ (18, 19), casein kinase 2 (20), or GSK-5 (21).

Purification of Glycogen Synthase Kinases—The major form of glycogen synthase kinase, designated GSK-A1, was purified from porcine renal cortex as described under "Experimental Procedures." GSK-A1 was purified approximately 1000-fold with recoveries of 1–3% of the glycogen synthase kinase activity of the initial extract. A second glycogen synthase kinase, designated GSK-A2, was purified to a lesser extent (Table I). These enzymes were used for the studies described below.

Sedimentation Coefficient, Stokes Radius, and Molecular Weight of GSK-A1—Sucrose density gradients were carried out on glycogen synthase kinase after purification through the Millipore filter concentration of the hydroxyapatite eluate as described under "Experimental Procedures." The sedimentation coefficient was calculated to be 4.0. The peak widths of the standards and glycogen synthase kinase activity were essentially the same. Since the hydroxyapatite eluate contained both GSK-A1 and GSK-A2, these results indicate that these two forms have similar sedimentation coefficients.

The Stokes radius of GSK-A1 was determined on a calibrated Sephadex G-150 column in the presence of 0.01% Brij 35. The detergent was included to stabilize the enzyme and was also present during the calibration of the column with protein standards. The Stokes radius was determined to be 2.85 nm (Table II). This value was not significantly different from the value of 2.95 nm obtained for the partially purified enzyme chromatographed on a Sephadex G-100 column in the absence of the detergent Brij 35 (23). When the value of 2.85 nm for Stokes radius and the sedimentation coefficient of 4.0 were used, the calculated molecular weight was 48,000 for GSK-A1 (Table II). This value is in good agreement with the apparent molecular weights of 45,000 for GSK-A1 determined from the calibrated Sephadex G-150 chromatography (Table II) and the prominent band obtained on SDS-PAGE (45,000).

TABLE II

Properties of GSK-A1

Gel filtration and sucrose density gradient centrifugation were carried out as described under "Experimental Procedure" and in the text. pH studies are described in the text.

Property	
Stokes radius (nm)	2.85
$s_{20,w}$	4.00
Molecular weight	
Gel filtration	45,000
Calculated	48,000
Frictional ratio	1.20
pH optimum	7.0–8.5

TABLE III

Protein substrate specificity of GSK-A1

The reaction was carried out as described under "Experimental Procedures" for the phosphorylation of glycogen synthase except the substrate was varied. Each substrate was at 0.1 mg/ml. Tris-HCl was 50 mM and β-glycerol phosphate was 9 mM. The ^{32}P-protein was isolated on Whatman ET-31 cellulose filter papers as previously described (25). Time courses were run in duplicate, and the rates were determined by regression analysis. The data are expressed as the mean ± S.E.

Substrate	Glycogen synthase kinase A1 activity
	pmol/min/μg
Synthase (glycogen-bound)	76 ± 2.6
Synthase (glycogen-free)	79 ± 5.7
Phosphorylase *b*	0.1 ± 0.5
Phosphorylase *b* kinase	3.6 ± 3.5
Histone (mixed)	0.8 ± 0.6
Phosvitin	4.9 ± 1.3
Casein	0.0 ± 2.1

Effect of pH on Activity of GSK-A1—The effect of pH on GSK-A1 activity was determined with MES buffer between pH 5.5 and 7.0 and Tris buffer between 7.0 and 8.5. The enzyme had a broad pH optimum with essentially the same activity between pH 7.0 and 8.5. There was a marked decrease in activity as the pH was decreased from pH 7.0 to 5.5.

Nucleotide Substrate Specificity of GSK-A1—Since some of the glycogen synthase kinases purified from other laboratories have been reported to use GTP (19–21) and dATP (25) as phosphoryl donors, it was of interest to determine the nucleotide specificity of GSK-A1. GSK-A1 inactivated glycogen synthase most effectively when 1 mM ATP was the phosphate donor. dATP was only about 15% as effective as ATP. GSK-A1 could not use 1 mM GTP, CTP, ITP, or UTP in place of ATP.

Protein Substrate Specificity of GSK-A1—Table III shows the protein substrate specificity of GSK-A1. Both glycogen-free and glycogen-bound glycogen synthase served equally well as substrates. GSK-A1 was unable to catalyze the phosphorylation of phosphorylase kinase when the substrate concentration was 0.1 (Table III) or 0.4 mg/ml (data not shown) or phosphorylase when the concentration was 0.1 (Table IV) or 19 mg/ml (data not shown). Histone, casein, and phosvitin have often been used to follow the purification of glycogen synthase kinase. These proteins were poor substrates for

GSK-A1 (Table III). GSK-A1 was unable to phosphorylate cGMP-dependent protein kinase, type II regulatory subunit, type I regulatory subunit of cAMP-dependent protein kinase (data not shown). In addition, GSK-A1 was unable to incorporate phosphate into 6-phosphofructo-1-kinase or 6-phosphofructo-2-kinase (data not shown). Therefore, GSK-A1 appears to be highly specific for glycogen synthase.

Potential Modifiers of GSK-A1 Activity—Several potential modifiers of GSK-A1 activity were tested. NaCl was found to be inhibitory and it was, therefore, important to remove salt before assaying glycogen synthase kinase fractions. 64 mM NaCl caused approximately 50% inhibition of GSK-A1. Other inhibitors of GSK-A1 activity included 1 mM Na_2SO_4 (55%), 0.5 mM UDP (30%), 0.5 mM UDP-glucose (35%), 0.5 mM ADP (62%), and 50 mM KF, the most effective inhibitor (95%). The sugar phosphates glucose-6-P, glucosamine 6-phosphate, and glucose-1-P (0.5 mM each) had no effect on GSK-A1 activity. Glycogen synthase kinase activity was not affected by 1 mM fructose 2,6-bisphosphate, 2 mM EGTA, 0.2 mM Ca^{2+} and 0.4 μM calmodulin, 5 μM cAMP, 5 μm cGMP, 80 μM trifluoperazine, or 5 μg of phosphatidylserine/ml in the presence of 0.2 mM Ca^{2+} and 0.4 μM calmodulin.

Kinetic Parameters of GSK-A1—The $s_{0.5}$ values for glycogen synthase and ATP were determined for GSK-A1. The kinetics for glycogen synthase were determined using the following as substrates: 1) glycogen-bound synthase; 2) glycogen-free synthase with 4 mg of glycogen/ml present; and 3) glycogen-free synthase. Assuming a tetrameric structure for glycogen synthase, the K_m values for GSK-A1 were 0.26, 0.46, and 0.33 μM, respectively. The Hill coefficients were not different from 1.0. The presence of glycogen had very little effect on the V_{max}. The K_m value using ATP as the variable substrate was determined to be 13.1 μM.

Phosphorylation and Inactivation of Glycogen Synthase by GSK-A1 and GSK-P—Phosphorylation and inactivation of glycogen synthase by several concentrations of GSK-A1 and GSK-P are shown in Figs. 3 and 4, respectively. Little if any change in the activity ratio was seen in the control experiment. When 70 phosphorylation units of GSK-A1/ml were present, phosphate incorporation reached a maximum of 0.5 mol of ^{32}P/mol of synthase subunit and did not change over the next 90 min of incubation (Fig. 3, *upper*). The activity ratio was reduced to about 0.75 (Fig. 3, *lower*). When the concentration of GSK-A1 (in units/ml) was 835, 1670 (Fig. 3), or 3000 (not shown), most of the reaction was completed within 3 min and after 2 h of incubation no more than 1 mol of ^{32}P/mol of synthase subunit was incorporated and the activity ratio was reduced to about 0.5.

Fig. 4 shows the same type of experiment with GSK-P. Forty phosphorylation units of GSK-P/ml catalyzed the incorporation of nearly 1 mol of ^{32}P/mol of synthase subunit in 60 min. When 1050 units of GSK-P/ml were incubated with synthase, nearly 2.5 mol of ^{32}P/mol of synthase subunit were incorporated and the activity ratio was reduced to less than 0.20. A similar concentration of GSK-A1 (835 units/ml) catalyzed incorporation of only 1 mol of ^{32}P/mol of synthase subunit and reduced the activity ratio to 0.50–0.60 (Fig. 3). The correlation of phosphorylation with activity ratio for GSK-A1 and GSK-P is illustrated in Fig. 5. GSK-A2 (600 units/ml) catalyzed phosphorylation of synthase to the level of 1.7 mol of ^{32}P/mol of synthase subunit and reduced the activity ratio to 0.3 (data not shown). Thus, it appears that kinases in GSK-P resolved by AEP-agarose chromatography have different specificities with respect to the phosphorylation sites on glycogen synthase.

Glycogen Synthase Phosphorylation Site Specificity—Glycogen synthase can be phosphorylated at multiple sites. Several approaches to the localization of the phosphorylation sites on the synthase molecule were attempted. The first approach used trypsin to partially digest the ^{32}P-labeled synthase. After limited trypsin digestion, two classes of peptides

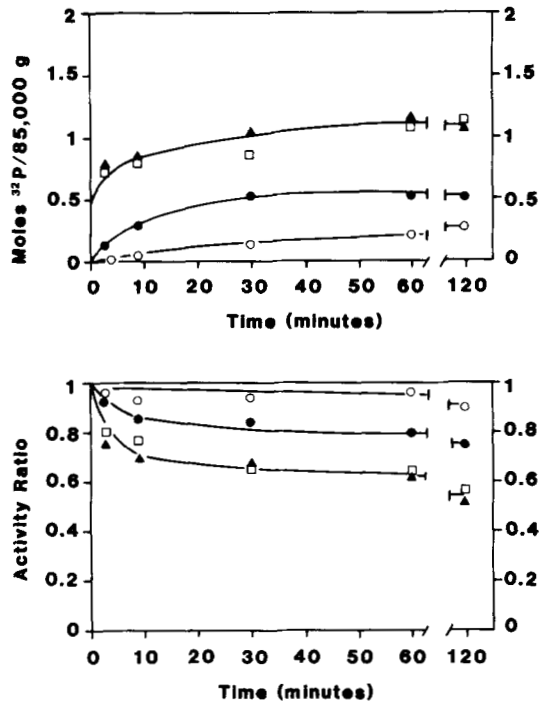

FIG. 3. **Time courses for the phosphorylation (*upper*) and inactivation (*lower*) of glycogen-bound glycogen synthase by GSK-A1.** GSK-A1 was added to yield a final concentration of 0 (○), 70 (●), 835 (□), and 1670 (▲) phosphorylation units/ml. Phosphorylation obtained in the absence of any added GSK-A1 was substracted to give net phosphorylation in the presence of kinase.

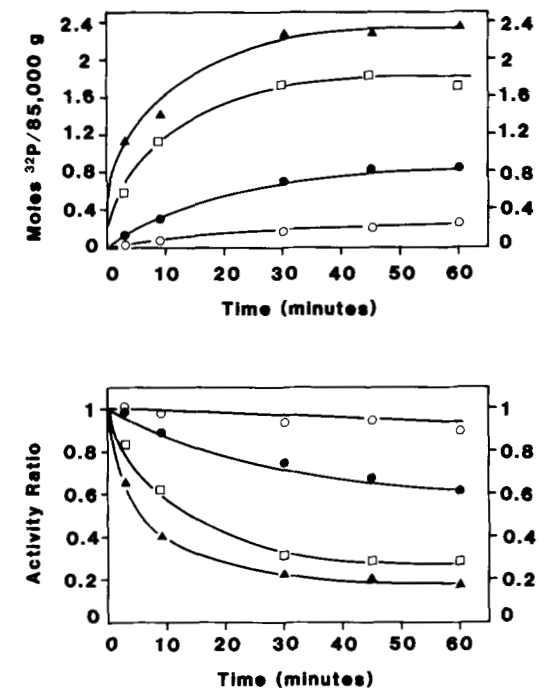

FIG. 4. **Time courses for the phosphorylation (*upper*) and inactivation (*lower*) of glycogen-bound glycogen synthase by GSK-P.** GSK-P was added to yield a final concentration of 0 (○), 40 (●), 210 (□), and 1050 (▲) phosphorylation units/ml. In the presence of GSK-P, net phosphorylation is presented.

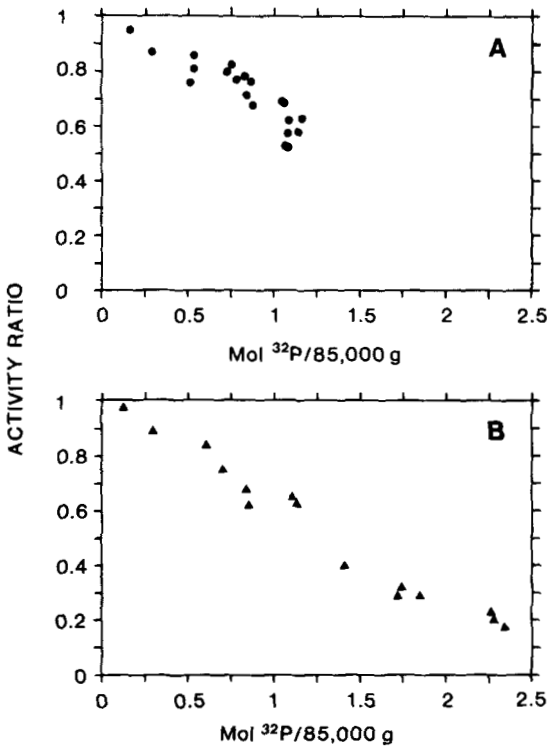

FIG. 5. **Correlation of glycogen synthase phosphorylation and inactivation catalyzed by GSK-A1 (*A*) and GSK-P (*B*).** The activity ratio was plotted as a function of the phosphorylation state using data obtained from experiments as described in Figs. 3 and 4.

TABLE IV

Distribution of phosphate between trypsin-sensitive and trypsin-insensitive regions of glycogen synthase

^{32}P-labeled glycogen synthase (glycogen-bound) was prepared to various degrees of phosphorylation with GSK-A1, GSK-P, or catalytic subunit of cAMP-dependent protein kinase. The determination of trypsin-sensitive and trypsin-insensitive regions of ^{32}P-labeled glycogen synthase was determined as previously described (40). The data (phosphates/subunit) are expressed as the mean ± S.E. (n = 4).

Kinase	Total ^{32}P	Trypsin-sensitive ^{32}P	Trypsin-insensitive ^{32}P	Trypsin-insensitive ^{32}P
				%
GSK-A1	0.22 ± 0.01	0.01 ± 0.01	0.21 ± 0.01	95
	0.38 ± 0.01	−0.04 ± 0.03	0.42 ± 0.02	111
	0.77 ± 0.05	0.03 ± 0.05	0.74 ± 0.00	96
GSK-P	0.33 ± 0.01	−0.01 ± 0.06	0.34 ± 0.04	103
	1.26 ± 0.07	−0.15 ± 0.03	1.41 ± 0.03	112
	1.88 ± 0.01	0.18 ± 0.05	1.70 ± 0.06	90
cAMP-dependent protein kinase	0.68 ± 0.05	0.58 ± 0.08	0.1 ± 0.03	15

are generated. These can be differentiated by their solubility in trichloroacetic acid (39) (Table IV). When synthase was phosphorylated to a level of 0.7 mol of ^{32}P/mol of synthase subunit by GSK-A1, essentially all of the phosphate was incorporated in the trypsin-insensitive site (trichloroacetic acid-insoluble). Likewise, when GSK-P catalyzed the transfer of 0.3, 1.3, or 1.9 mol of ^{32}P/mol of synthase subunit, by and large all of the ^{32}P was associated with trypsin-insensitive sites. Since GSK-P contains a mixture of kinases, it appeared that all of these forms had a high specificity for the trypsin-insensitive phosphorylation domains. In contrast, the first phosphate incorporated into synthase by the catalytic subunit

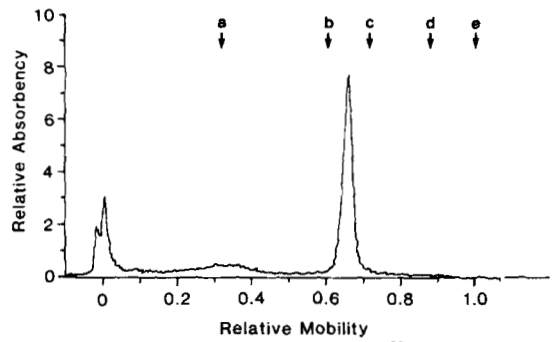

FIG. 6. **SDS-PAGE of CNBr-cleaved ^{32}P-labeled glycogen synthase.** The glycogen synthase was phosphorylated to a level of 0.8 ml of ^{32}P/mol of synthase subunit. After CNBr cleavage, as described by Soderling *et al.* (40), the sample was subjected to SDS-PAGE as described under "Experimental Procedures." The gels were exposed to film and the resulting autoradiogram was scanned on a densitometer. The protein standards and molecular weights include: *a*, chymotrypsinogen (25,700); *b*, lysozyme (13,300); *c*, bovine trypsin inhibitor (6,000); *d*, insulin (3,000), and *e*, the dye front.

of cAMP-dependent protein kinase was found to be in the trypsin-sensitive domain (Table IV).

A second approach was to use CNBr to cleave the ^{32}P-labeled synthase molecule (40). Synthase was phosphorylated to the level of 0.80 mol of ^{32}P/mol of a subunit with GSK-A1. After cleavage with CNBr, the synthase was then electrophoresed on SDS-PAGE. The gels were then exposed to film and the resulting autoradiogram was scanned on a densitometer (Fig. 6). The scan indicated that the major phosphorylated site was on a low molecular weight peptide (approximately 10,000). Cohen *et al.* (21) have recently shown that when ^{32}P-labeled glycogen synthase is digested by low concentrations of trypsin, sites 2, 3a, 3b, and 3c are in the trypsin-insensitive domain. In addition, Cohen *et al.* indicated that the low molecular weight CNBr peptide contained only site 2 (*i.e.* Ser-7). Consequently, since the GSK-A1 synthase phosphorylation site is trypsin-insensitive and on a low molecular weight CNBr peptide, it appears that GSK-A1 phosphorylates site 2. This was confirmed by the use of reverse phase high performance liquid chromatography of tryptic digests. When GSK-A1 incorporated 0.80 mol of ^{32}P/mol of synthase subunit, 95% of the label eluted in a position corresponding to site 2 (Fig. 7). The small peak eluting near 115 min and preceding the major peak appears to be a breakdown product of site 2.[2]

To further establish that Ser-7 was the site phosphorylated by GSK-A1, glycogen synthase was phosphorylated by GSK-A1 to the extent of 0.7 mol of ^{32}P/mol of subunit. The ^{32}P-synthase was then incubated under standard phosphorylation conditions with phosphorylase kinase, an enzyme specific for Ser-7 (21). Aliquots were removed at 15, 30, and 45 min and ^{32}P bound to synthase was determined. There was no additional phosphorylation of synthase by the added phosphorylase kinase (data not shown). When glycogen synthase was incubated in an identical manner except GSK-A1 was not added, subsequent addition of phosphorylase kinase resulted in the incorporation of 0.4 mol of ^{32}P/mol of synthase subunit within 15 min. Phosphorylation of glycogen synthase by phosphorylase kinase leveled off at about 0.5 mol of ^{32}P/mol of subunit. Conversely, if glycogen synthase was first phosphorylated by phosphorylase kinase and then incubated with GSK-A1, little additional phosphate was incorporated into synthase.

[2] T. R. Soderling, personal communication.

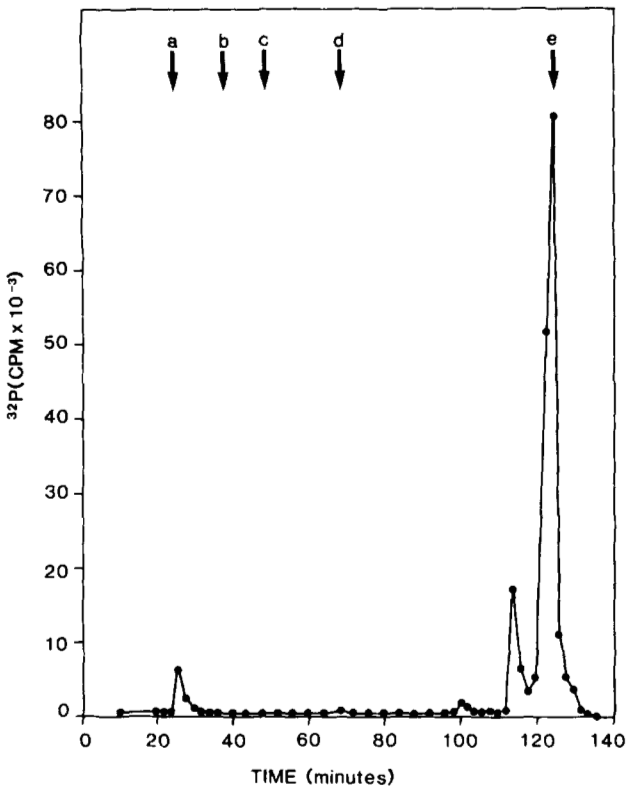

FIG. 7. **Reverse phase high performance liquid chromatography of ^{32}P-labeled glycogen synthase tryptic peptides.** The procedure was carried out according to the method of Juhl *et al.* (35). Glycogen synthase was phosphorylated to a level of 0.7 mol of ^{32}P/mol of synthase subunit with GSK-A1 and then precipitated with 10% trichloroacetic acid. The precipitate was washed twice with ether and taken up in 100 mM ammonium bicarbonate (pH 8.0) and then digested with 1 mg/ml of trypsin for 5 h. The digest was then chromatographed on a Beckman 421 high performance liquid chromatograph using an acetonitrile gradient in 0.1% trifluoroacetic acid on a Beckman C-18 Ultrasphere ODS column (0.46 × 25 cm). The gradient flow rate was 1 ml/min as follows: 0.5% acetonitrile for 10 min, 5–38% for 100 min, and 38–50% for 5 min. Two-ml fractions were collected. ^{32}P was determined by Cerenkov's counting. The *arrows* indicate the elution positions: *a*, site 1a; *b*, site 5; *c*, sites 3abc; *d*, site 1b; *e*, site 2.

DISCUSSION

Multiple forms of cAMP- and Ca^{2+}-calmodulin-independent glycogen synthase kinases have been previously demonstrated in rabbit skeletal muscle (18, 21, 41), rat skeletal muscle (25), and several other rat tissues (22). Schlender and Reimann (22, 24) subdivided glycogen synthase kinases into forms that bind to phosphocellulose (GSK-P) and forms that bind to DEAE-cellulose (GSK-D). Schlender *et al.* (25) used Sephadex G-100 to separate two forms of GSK-P from rabbit skeletal muscle. DePaoli-Roach *et al.* (18) further subdivided synthase kinases into forms that elute from phosphocellulose at low ionic strength ($PC_{0.4}$ and $PC_{0.5}$) and forms that elute at high ionic strength ($PC_{0.7}$). Cohen *et al.* (21), using a procedure similar to that of DePaoli-Roach *et al.* (18), have recently separated three distinct glycogen synthase kinases designated GSK-3, GSK-4, and GSK-5. The latter kinase appears to be the phosvitin/casein kinase designated $PC_{0.7}$ by DePaoli-Roach *et al.* (18, 19) or casein kinase 2 by Huang *et al.* (20). This kinase differs from the other glycogen synthase kinases studied in that it phosphorylates but does not inactivate glycogen synthase.

Several lines of evidence are presented here which demonstrate that porcine renal cortex contains multiple forms of cyclic nucleotide- and Ca^{2+}-calmodulin-independent glycogen synthase kinases. The major glycogen synthase kinase forms in crude extracts were bound to phosphocellulose and eluted at low ionic strength (GSK-P), while a lesser form, GSK-D, failed to bind to the phosphocellulose. Since GSK-D appeared to be slightly larger than GSK-P (50,000–55,000 *versus* 45,000–50,000), it is possible that the loss of a small peptide may differentiate these two forms. Although protease inhibitors had no effect on the appearance of multiple forms of glycogen synthase kinase, it is possible that these were generated by resistant proteases or by a nonproteolytic mechanism. Since renal cortex contains a heterogeneous population of cells, it is also possible that these forms originate from different cell types. These activities were determined using the conversion assay which was used to detect only kinases which inactivate glycogen synthase and have low K_m values for this substrate. Consequently, the phosphorylation assay was required to detect the heparin-sensitive phosvitin/casein kinase which phosphorylates but does not inactivate glycogen synthase (18–21).

We have previously reported on the partial purification of GSK-P from porcine renal cortex (23). Although those data suggested a homogeneous preparation with respect to kinase, subsequent purification demonstrated GSK-P to be a heterogeneous group of glycogen synthase kinases. These kinases were unresolvable by procedures previously utilized including successive salt step elutions or gradient elution from phosphocellulose, gradient elution from CM-cellulose, DEAE-cellulose, or gel filtration. Only ATP gradient elution from AEP-agarose was capable of separating multiple forms of renal GSK-P. Much higher concentrations of sodium chloride or phosphate were required to elute glycogen synthase kinase activity from AEP-agarose and there was no separation of forms.[3] When crude extracts were directly chromatographed on AEP-agarose, about 70% of the glycogen synthase kinase activity eluted at the same position as GSK-A1.[3] This suggests that GSK-A1 was not generated by the purification procedure. It is interesting that GSK-A1 and the other kinases comprising GSK-P readily bound to resins containing phosphate groups. GSK-P bound tightly to phosphocellulose but poorly to CM-cellulose. Since both sodium chloride and sodium citrate were required for elution, the behavior of these kinases on hydroxyapatite suggests that they bound to the calcium site as well as to the phosphate site.

The most remarkable feature of GSK-A1 is its specificity for glycogen synthase and ATP. In addition, GSK-A1 catalyzed phosphorylation of only one site at or near Ser-7 at the NH_2 terminus of the molecule. Several separate pieces of evidence confirm this finding. Only 1 mol of ^{32}P/mol of subunit was incorporated into glycogen synthase. This phosphate was located in a M_r = 10,000–12,000 CNBr peptide of glycogen synthase and in a trypsin-insensitive peptide of glycogen synthase. Glycogen synthase phosphorylated with GSK-A1 could not be further phosphorylated by phosphorylase kinase. These observations are consistent with GSK-A1 catalyzing the phosphorylation of glycogen synthase at Ser-7 (site 2) (21). High performance liquid chromatography, done in collaboration with Dr. Thomas R. Soderling, was a final piece of evidence demonstrating that GSK-A1 only catalyzed the phosphorylation of a site tentatively identified as Ser-7 (site 2). Although the only known phosphorylation site near the NH_2 terminus of glycogen synthase is Ser-7, we have not ruled out at this time that phosphorylation occurs at another

[3] S. J. Beebe, E. M. Reimann, and K. K. Schlender, unpublished results.

site near Ser-7 on glycogen synthase. This substrate specificity for synthase but not phosphorylase or phosphorylase kinase suggests that GSK-A1 is also specific in its role in glycogenesis. In similar studies GSK-A2 was found to evenly distribute about 2 mol of ^{32}P/mol of synthase monomer into sites 1a, 2, and 3a, b, c.[3] It should be noted that these studies used rabbit muscle glycogen synthase as substrate. It is not known if the phosphorylation of renal glycogen synthase would be identical.

Phosphorylase kinase (21), Ca^{2+}-calmodulin-dependent glycogen synthase kinase (15–17), and GSK-4 (21) are other kinases which have been shown to catalyze the phosphorylation of Ser-7 on glycogen synthase. Since GSK-A1 did not phosphorylate phosphorylase and was not stimulated by Ca^{2+}-calmodulin or inhibited by EGTA or trifluoperazine, this kinase cannot be identified as phosphorylase kinase or the Ca^{2+}-calmodulin-dependent glycogen synthase kinase. GSK-A1 and GSK-4 (21) were similar in that both were eluted from phosphocellulose at low ionic strength and have low Michaelis constants and are specific for glycogen synthase and ATP. However, GSK-4 bound to DEAE-cellulose and was eluted with 0.2 M NaCl, while repeated attempts to use DEAE-cellulose to purify GSK-A1 indicated it would not bind under the most favorable conditions. In addition, GSK-A1 was inhibited 50% by 64 mM NaCl while GSK-4 was stimulated by a similar NaCl concentration (21). Based on gel filtration, the apparent molecular weight was 45,000–50,000 for GSK-A1 and 115,000 for GSK-4. Finally, GSK-A1 and the other kinases in GSK-P were not stable for extended periods of time in the Tris buffers used to purify GSK-4. Thus, it is clear that major differences exist between GSK-A1 and GSK-4.

The glycogen synthase activity ratio in rabbit kidney cortex is low (0.05 and the $A_{0.5}$ for glucose-6-P is high (2 mM) (23). These two phenomena can be explained by extensive phosphorylation of cortical glycogen synthase *in vivo*. Schlender and Reimann (22) demonstrated that 80–95% of the glycogen synthase kinase activity in rat kidney was independent of cAMP. Also, 70–80% of the glycogen synthase kinase activity of rabbit renal cortex or medulla was insensitive to the heat-stable inhibitor of cAMP-dependent protein kinase (23). Mendicino *et al.* (42) determined that the apparent K_m of the cAMP-dependent protein kinase for glycogen synthase (50 μM) was about 1300 times the kidney glycogen synthase concentration. These data suggest that the cAMP-dependent protein kinase plays a modest role in the inactivation of renal synthase. Since no Ca^{2+}-calmodulin-stimulated glycogen synthase kinase activity could be detected in renal cortex extracts (23), it also appears that Ca^{2+}-calmodulin synthase kinase does not play an important role in producing the highly inactivated and phosphorylated glycogen synthase in kidney cortex. Given the predominance of cAMP-independent glycogen synthase kinase in rabbit renal cortex (23, 24) and GSK-A1 in swine renal cortex, it is highly likely that this class of kinases plays an important role in the regulation of glycogen synthase in renal cortex. Although the regulatory mechanism of these kinases has not yet been determined, it is possible that control is afforded by known synthase effectors (UDP, UDP-glucose, ADP, and Na_2SO_4) (43) which caused some inhibition of GSK-A1 or by some as yet undefined regulators. This regulation could occur by an allosteric modulation at the level of the substrate and/or kinase.

It appears that more than one kinase participates in the inactivation of renal cortical glycogen synthase. GSK-A1 catalyzed the transfer of only 1 mol of ^{32}P/mol of synthase subunit and reduced the activity ratio to about 0.5. GSK-A2 catalyzed the transfer of a 2nd mol of ^{32}P/mol of synthase subunit, but reduced the activity ratio to only 0.30. When both GSK-A1 and GSK-A2 were combined, synthase was phosphorylated and inactivated to the same degree as with GSK-A2 alone. However, the GSK-P preparation catalyzed the phosphorylation of at least three sites and reduced the activity ratio to $\leq$0.10. This suggests that still another kinase, a kinase activator, or substrate modulator is present in the GSK-P preparation.

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Purification and characterization of a cAMP- and Ca2+-calmodulin-independent glycogen synthase kinase from porcine renal cortex.

S J Beebe, E M Reimann and K K Schlender

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