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ISOLATION AND CHARACTERIZATION OF CYCLIC AMP-INDEPENDENT GLYCOGEN SYNTHASE KINASE FROM RAT SKELETAL MUSCLE

KEITH K. SCHLENDER [a], STEPHEN J. BEEBE [a], JAMES C. WILLEY [a], STEPHEN A. LUTZ [a] and ERWIN M. REIMANN [b]

[a] *Department of Pharmacology and Therapeutics and* [b] *Departments of Biochemistry and Surgery, Medical College of Ohio, Toledo, OH 43699 (U.S.A.)*

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Summary

Glycogen synthase kinase was isolated from rat skeletal muscle. This kinase, which is cyclic nucleotide-independent and calcium-independent, was separated from phosphorylase kinase, cyclic AMP-dependent protein kinase and phosvitin kinase by phosphocellulose chromatography. Gel filtration on Sephadex G-100 resolved the glycogen synthase kinase into two fractions with apparent molecular weights of 68 000 (peak I) and 52 000 (peak II). This step also separated glycogen synthase kinase from the catalytic subunit of the cyclic AMP-dependent protein kinase, which had an apparent molecular weight of 39 000.

Peak II glycogen synthase kinase activity was not affected by the addition of calcium, EGTA or a number of cyclic nucleotides. In addition to ATP, dATP would serve as the phosphate donor. Other trinucleotides tested were either poor or ineffective substrates. Activity was about 5-fold greater with Mg^{2+} than with Mn^{2+}. Glycogen stimulated activity about 25%.

Modifications of the methods of Soderling et al. ((1970) J. Biol. Chem. 245, 6317—6328) and Nimmo et al. ((1976) Eur. J. Biochem. 68, 21—30) were developed for purification of glycogen synthase (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) to a specific activity of 35 units/mg of protein. Using this preparation of glycogen synthase as substrate, the phosphorylation and inactivation catalyzed by glycogen synthase kinase was compared to that catalyzed by cyclic AMP-dependent protein kinase or phosphorylase

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Abbreviations: glycogen synthase I, glucose-6-*P*-independent form of glycogen synthase; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid

The term glycogen synthase kinase is used to denote cyclic AMP-independent and calcium-independent kinases which phosphorylate and inactivate glycogen synthase.

kinase. Each of the kinases had different specificities for phosphorylation sites on glycogen synthase.

Introduction

Phosphorylation of glycogen synthase (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) converts the enzyme to forms which require the presence of glucose-6-*P* for full activity [1]. Glycogen synthase can accept at least four [2,3] phosphates per 85 000 dalton subunit. The relationship between degree of phosphorylation and dependence on glucose-6-*P* is complex but it is clear that enzyme activity is influenced by the degree of phosphorylation at two or more of these sites [3—5]. The kinases which act on glycogen synthase include the cyclic AMP-dependent protein kinase [6,7] and a group of kinases which are not sensitive to cyclic nucleotides [8—18]. The relationship between the different forms of the cyclic nucleotide-independent kinases has not been resolved. Several laboratories have shown that protein kinases prepared from rabbit skeletal muscle can phosphorylate and inactivate glycogen synthase [10,11,13,15,17]. It is not clear if these preparations represented major forms of glycogen synthase kinase in skeletal muscle, since purification was monitored by phosvitin [10,13,15] or casein [11,17] phosphorylation. Itarte and Huang [17] presented evidence that casein and phosvitin kinase activities are intrinsic to glycogen synthase kinase and are not the result of contaminating kinases. Soderling et al. [12] purified a glycogen synthase kinase from rabbit skeletal muscle which could be partially separated from both phosvitin kinase and casein kinase and concluded that phosphorylation of casein and phosvitin was catalyzed by contaminating kinases. In a previous study [16] we found that the major glycogen synthase kinase-fraction from rat skeletal muscle was not associated with the major phosvitin kinase. The phosvitin kinase from rat [16] or rabbit [18] skeletal muscle is relatively ineffective regarding inactivation of glycogen synthase.

Recently it has been established that phosphorylase kinase can also phosphorylate glycogen synthase [19—23]. The rate of phosphorylation of glycogen synthase is similar to that of phosphorylase [20—22] and the reaction rate is increased by the addition of Ca^{2+} [19—23] and the Ca^{2+}-dependent regulator protein [21,23].

Because a major portion of the glycogen synthase kinase activity in rat skeletal muscle is not cyclic nucleotide-dependent [16], it was of interest to characterize this enzyme in more detail and to differentiate it from phosphorylase kinase and cyclic AMP-dependent protein kinase. This report describes some of the properties of this form of glycogen synthase kinase which was purified from rat skeletal muscle. Enzyme purification was monitored by the ability of the kinases to catalyze inactivation of glycogen synthase, the presumed function of the enzymes in vivo. This approach avoids the potential pitfall of isolating a kinase which is relatively nonspecific in catalyzing inactivation of glycogen synthase. Some of these results have been reported in abstract form [24]. While this manuscript was in preparation, DePaoli-Roach et al. [18] reported some of the same observations using rabbit muscle kinases.

Methods

Materials

Oyster glycogen (type II) obtained from Sigma Chemical Co., was purified by mixed bed ion exchange chromatography [25]. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman, Inc. UDP [U-^{14}C]-glucose was obtained from ICN. [γ-^{32}P]GTP was obtained from Amersham/Searle. [γ-^{32}P]ATP was obtained commercially or prepared by the method of Walseth and Johnson [26]. Gelatin was purchased from BBL division of Becton, Dickinson, and Co., and trypsin (code: TRTPCK) from Worthington Biochemical Corporation. Poly(ethylene glycol) (approx. M_r 6000) and other reagents were obtained from Sigma Chemical Co.

Catalytic subunit of the cyclic AMP-dependent protein kinase with a specific activity of 1000 pmol ^{32}P incorporated into histone/min per μg protein was purified as previously described [27]. The enzyme was isolated from pig gastric mucosa but there is no evidence that the properties of the catalytic subunit of the cyclic AMP-dependent protein kinase from various tissues are different [28]. The heat-stable inhibitor of this kinase was isolated from rabbit skeletal muscle as described previously [16]. Rabbit skeletal muscle phosphorylase kinase was purified according to the method of Cohen [29] through the Sepharose 4B column step. Phosphorylase *b* (specific activity 70 units/mg protein) was prepared from rabbit skeletal muscle by the method of Fischer and Krebs [30]. Bovine brain Ca^{2+}-dependent regulator protein [31] was a gift from Dr. Charles Brostrom. Human salivary α-amylase was purified as previously described [32] through the Sephadex G-25 column step, lyophilized, and stored at 4°C.

Purification of glycogen synthase

The procedure for preparing glycogen synthase I was adapted from the methods of Soderling et al. [7] and Nimmo et al. [33]. Unless otherwise indicated, all operations were carried out at 4°C. In a typical preparation, 1.2 kg of fresh rabbit skeletal muscle were homogenized in 3 l of 4 mM EDTA, pH 6.8, and centrifuged at 10 000 × *g* for 45 min. The pH of the supernatant was adjusted to 6.1 with 1 N acetic acid and 10 min later the precipitate was collected by centrifugation at 10 000 × *g* for 20 min. A Dounce homogenizer was used to suspend the resulting protein/glycogen pellets in 240 ml of 50 mM potassium phosphate/1 mM EDTA (pH 7.5). After centrifugation at 78 000 × *g* for 90 min the resulting pellets were suspended in 72 ml of 50 mM Tris-HCl/1 mM EDTA (pH 7.5), at 4°C (buffer A). The suspension was either used immediately or stored at −70°C for 1—7 days before use. The suspension was centrifuged 78 000 × *g* for 90 min and the resulting pellets were suspended in 30 ml of buffer A containing 43 mM β-mercaptoethanol, 10 mM magnesium acetate, and 50 mg sucrose/ml. After adding 3.6 mg lyophilized human salivary α-amylase the suspension was incubated 1 h at 30°C to digest glycogen and to allow dephosphorylation of glycogen synthase by phosphatases. The preparation was centrifuged at 35 000 × *g* for 30 min and the supernatant was applied to a DEAE-cellulose column (2.6 × 6 cm) equilibrated in buffer A containing 43 mM β-mercaptoethanol and 10% glycerol. The enzyme was eluted with a linear gradient of 50—500 mM Tris-HCl in the same buffer. Fractions were

assayed for glycogen synthase and the peak fractions (about 150 to 250 mM Tris-HCl) were combined. The leading fractions were sometimes discarded to minimize contamination by kinases and phosphatases. 0.11 vol. of 50% poly(ethylene glycol) (w/v) was added and the sample was incubated 1 h at room temperature. Insoluble proteins were removed by centrifugation at 15 000 × *g* for 15 min and 0.11 vol. of 50% poly(ethylene glycol) was added to the supernatant. After overnight incubation at 4°C, the precipitated enzyme was collected by centrifugation at 15 000 × *g* for 20 min. The pellets were dissolved with the aid of a glass teflon tissue homogenizer in 2 ml of 33 mM β-glycerophosphate/0.67 mM EDTA/29 mM β-mercaptoethanol/33% glycerol, pH 7.0 (buffer B). About 20 mg of glycogen free enzyme (i.e. no activity in the absence of added primer glycogen) could be prepared in 3 days from 1.2 kg muscle. At this stage the enzyme could be stored for several months at −70°C with little loss of activity. The synthase had an activity ratio of about 0.95, a specific activity of about 14 units/mg protein, a sedimentation coefficient of 14 S, and behaved predominantly as a single band with a molecular weight of 85 000 when subjected to polyacrylamide gel electrophoresis in the presence of SDS (See Fig. 5A, Ref. 21).

The enzyme preparation was nearly free of glycogen synthase kinase activity except for varying amounts of phosphorylase kinase [21]. The contaminating phosphorylase kinase was either negated by adding 0.175 mM EGTA to the kinase reaction or removed by phosphocellulose chromatography [22]. In a typical experiment approx. 1 mg enzyme was applied to a 2 ml phosphocellulose column equilibrated with buffer B. Approx. 60% of glycogen synthase and 1% of the phosphorylase kinase applied was recovered in the flow-through fraction. Over 90% of the applied phosphorylase kinase could be eluted from the column with buffer B containing 1 M NaCl.

To prepare glycogen-bound synthase, glycogen-free synthase was diluted to a concentration of 1 unit/ml in 5 mM Tris-HCl/1 mM EDTA/43 mM β-mercaptoethanol/1 mg glycogen/ml (pH 7.5 at 4°C). The solution was stirred for 5 min at 0°C, then ethanol chilled to −70°C was slowly added while maintaining the temperature at 0—2°C with an ice-salt bath. After the concentration of ethanol reached 15% the solution was stirred for 15 min maintaining the temperature at −4 -5°C. Temperature control was important since the synthase-glycogen complex was not precipitated at temperatures above 2°C. The synthase-glycogen complex was collected by centrifugation at 10 000 × *g* for 20 min at −5°C and dissolved in 2 ml buffer B. After 1 or 2 precipitations with ethanol the specific activity increased to about 35 units/mg protein with an average yield of 110%. The increase in specific activity was probably due partly to activation by glycogen (see below) and partly due to removal of proteins, including denatured glycogen synthase, which do not bind to glycogen. The synthase-glycogen complex had a sedimentation coefficient of 25 S and like the glycogen-free enzyme showed one major band with a molecular weight of 85 000 when electrophoresed in the presence of SDS. The enzyme could be stored at −70° C for several months without loss of activity.

Glycogen synthase kinase assay

Glycogen synthase kinase activity was determined by measuring the inactiva-

tion of glycogen-bound glycogen synthase I as previously described [16] except that the reaction mixture differed. The reaction mixture (100 μl) contained 0.1 unit glycogen synthase/ml, 17.5 mM β-glycerophosphate (pH 7.0), 0.9 mM EDTA, 7 mg glycogen/ml, 1 mg gelatin/ml, 30 mM β-mercaptoethanol, 2 or 20 mM potassium phosphate, 0—40 mM NaCl, 5 mM ATP, 7 mM $MgCl_2$ and, when present, 50 mM KF and 5 μM cyclic AMP. A unit of glycogen synthase kinase activity is defined as the amount of enzyme which, during a 30 min incubation at 30°C causes the glycogen synthase I activity to decrease by 1 munit [16].

Phosphorylation and inactivation of glycogen synthase

Glycogen synthase was phosphorylated at 30°C in a reaction mixture containing 0.1 mg glycogen-bound glycogen synthase/ml, 17 mM β-glycerophosphate (pH 7.0), 0.7 mM EDTA, 1.75 mM EGTA, 4 mg glycogen/ml, 0.6 mg gelatin/ml, 2% glycerol, 20 mM potassium phosphate, 40 mM NaCl, 17 mM β-mercaptoethanol, 0.2 mM [γ-^{32}P]ATP (approx. 50 cpm/pmol) and 10 mM magnesium acetate. The reaction was initiated by the addition of ATP and magnesium acetate. At various time points, aliquots were removed and analyzed for protein-bound ^{32}P [34]. At the same time points, aliquots were diluted to 0.15 units glycogen synthase per ml in 50 mM Tris-HCl/10 mM EDTA/50 mM KF/10 mg glycogen/ml/43 mM β-mercaptoethanol (pH 7.5). Glycogen synthase activity was then determined in the absence and in the presence of glucose-6-*P* as described elsewhere [25]. Activity in the presence of glucose-6-*P* usually remained constant during the kinase reaction. Activity ratio is defined as the activity in the absence of glucose-6-*P* divided by the activity in the presence of glucose-6-*P*.

Protein kinase assays

Protein kinase activity was determined by measuring incorporation of the γ-phosphate of [γ-^{32}P]ATP or [γ-^{32}P]GTP into the indicated protein. Unless otherwise indicated, the conditions were as described for glycogen synthase phosphorylation except that glycogen synthase was replaced by the appropriate protein substrate at the indicated concentration. [^{32}P]Histone and [^{32}P]protamine were isolated on Whatman P-81 phosphocellulose paper [16], while other [^{32}P]proteins were isolated on Whatman ET-31 cellulose paper [34]. A unit of protein kinase activity is defined as that amount of enzyme which catalyzes the transfer of 1 pmol phosphate to substrate per min at 30°C.

Results

Activation of glycogen synthase by glycogen

When glycogen-free synthase was assayed under standard reaction conditions [25] either in the presence or absence of glucose-6-*P*, the time course exhibited a lag suggesting a time-dependent activation of the enzyme during the assay (Fig. 1). The glycogen-bound synthase gave a linear time course (data not shown). A similar hysteretic response of glycogen-free synthase I prepared from human polymorphonuclear leukocytes has been shown to be due to activation by glycogen [35]. Likewise, Nimmo et al. [33] reported that skeletal muscle

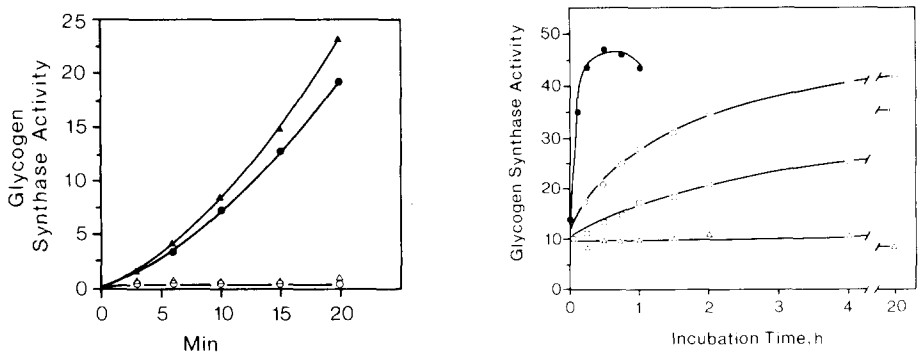


Fig. 1. Time course of glycogen synthase activity. Glycogen synthase (10 μg/ml) purified through the poly(ethylene glycol) step was preincubated at 30°C for 30 min with 25 mM β-glycerophosphate/1 mM EDTA/43 mM β-mercaptoethanol/1.5 mg gelatin/ml (pH 7.0). The enzyme was assayed for synthase activity [25] without glycogen (open symbols) or with 6.7 mg glycogen/ml (closed symbols) in the absence of (○, ●) or presence of 7.2 mM glucose 6-*P* (△, ▲). Glycogen synthase activity is expressed as nmol glucose added to glycogen per 75 μl reaction mixture. Similar results were obtained when β-glycerophosphate was replaced with 50 mM Tris-HCl (pH 7.8 at 30°C).

Fig. 2. Effect of ethanol on the activation of glycogen synthase by glycogen at 0°C. Glycogen synthase (40 μg/ml) purified through the poly(ethylene glycol) step was incubated on ice in a buffer containing 50 mM Tris-HCl, 5 mM EDTA, 43 mM β-mercaptoethanol (pH 8.3 at 0°C) with no additions (△———△), with 1 mg glycogen/ml (□———□), with 10 mg glycogen/ml (○———○) or with 10 mg glycogen/ml + 10% ethanol (●———●). At the appropriate time aliquots were removed and assayed for glycogen synthase activity [25] for 2 min. Glycogen synthase activity is expressed as nmol glucose added to glycogen in 2 min per 75 μl reaction mixture.

glycogen synthase which had been prepared glycogen-free by phosphorolysis was activated when incubated for 12—24 h at 4°C with glycogen. When glycogen-free synthase was incubated on ice with 10 mg glycogen/ml, there was an activation of synthase which reached a maximum at about 4 h (Fig. 2). The rate of activation was less at 1 mg glycogen/ml. The inclusion of 10% ethanol greatly increased the rate but not the extent of activation observed in the presence of 10 mg glycogen/ml. Ethanol (10%) alone had no effect on synthase activity (data not shown). Thus, in the preparation of the glycogen-bound enzyme, ethanol probably accelerates both the formation and the precipitation of the synthase-glycogen complex. Because the glycogen-free enzyme had a non-linear time course, the glycogen-bound enzyme was used as the substrate for the glycogen synthase kinase assays.

Purification of glycogen synthase kinase

Approx. 50 g muscle were removed from the hind leg of decapitated Sprague-Dawley rats (300—500 g) and chilled on ice. All subsequent operations were at 4°C. The tissue was homogenized with 2 ml of 5 mM K_2HPO_4/5 mM KH_2PO_4/1 mM EDTA (buffer C)/g of tissue in a Sorvall Omnimixer. The homogenate was centrifuged at 30 000 × *g* for 20 min. The supernatant was poured through glass wool and then applied to the phosphocellulose column (2.6 × 4.7 cm) equilibrated with buffer C. The column was washed extensively with buffer C and then with several bed volumes of buffer C which contained 0.5 M NaCl. The major glycogen synthase kinase was eluted in the 0.5 M NaCl fraction (Fig. 3). Activity could be detected by both glycogen synthase phos-

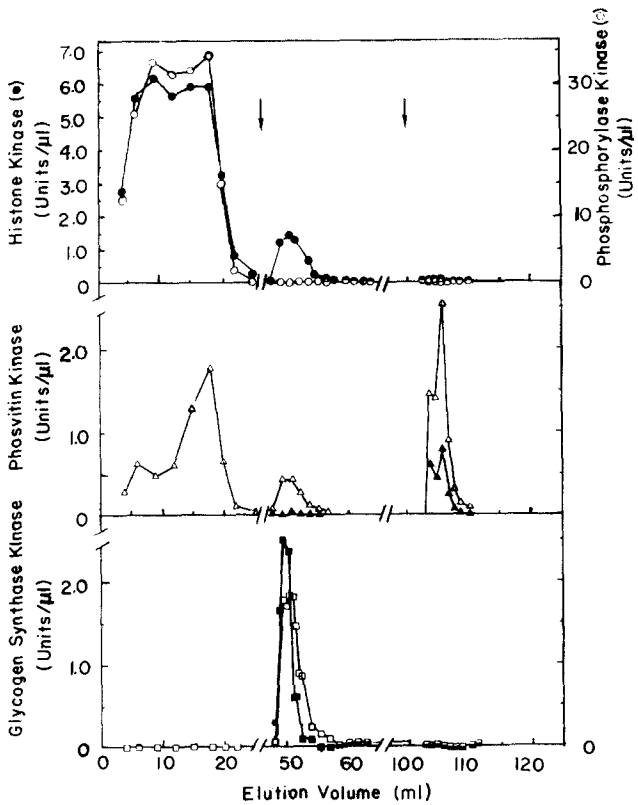


Fig. 3. Phosphocellulose chromatography of a rat skeletal muscle extract. An extract was prepared from 10 g tissue and applied to a phosphocellulose column (1.6 × 2.5 cm) which was equilibrated with 5 mM K_2HPO_4/5 mM KH_2PO_4/1 mM EDTA (buffer C). After the sample was applied, the column was washed extensively with buffer C. Proteins were eluted by washing the column with buffer C containing 0.5 M NaCl (first arrow) followed by buffer C containing 1.5 M NaCl (second arrow). Fractions were assayed for inactivation of glycogen synthase in the presence of cyclic AMP and KF (□———□) and for phosphorylation of glycogen synthase (■———■). Histone (●———●) and phosvitin (△, ▲) kinase activity was assayed as described in the text except that 0.5 mM EGTA was added and that phosvitin phosphorylation was measured with 3 mM magnesium acetate and either ATP (△) or GTP (▲) as the phosphoryl donor. The concentration of histone and phosvitin was 6 mg/ml. Phosphorylase kinase (○———○) activity was measured at pH 8.2 with 0.2 mM $CaCl_2$, 1 mg phosphorylase *b*/ml and 10 μg Ca^{2+}-dependent regulator protein/ml as described previously [21].

phorylation and inactivation. In other experiments when the kinase was eluted with a linear NaCl gradient, the enzyme eluted at an ionic strength of less than 0.4 (data not shown). The recovery of glycogen synthase kinase activity from the column was usually 200—400% of the activity in the extract, indicating that interfering substances were removed in this step. These interfering substances were probably present in the flow-through fractions since in some experiments no glycogen synthase kinase activity could be detected in these fractions despite the presence of both phosphorylase kinase and cyclic AMP-dependent protein kinase.

Most (80—90%) of the histone kinase appeared in the flow-through fraction. This histone kinase activity was dependent upon cyclic AMP and was inhibited by the heat-stable inhibitor, indicating that it was due to the cyclic AMP-depen-

dent protein kinase. A small fraction of histone kinase was adsorbed to the column and eluted with 0.5 M NaCl. This fraction was also sensitive to the heat-stable inhibitor and subsequent studies (see below) showed that most of this activity was due to the catalytic subunit of the cyclic AMP-dependent protein kinase. All of the phosphorylase kinase activity appeared in the flow-through fraction and was therefore separated from glycogen synthase kinase. The recovery of phosphorylase kinase exceeded 100%, indicating the presence of an inhibitor in the extract.

For the experiment depicted in Fig. 3, an additional wash with buffer C which contained 1.5 M NaCl was included in order to elute phosvitin kinase [10,13,16,18]. Phosvitin kinase activity was found in all three fractions but only a small amount was eluted by 0.5 M NaCl. The phosvitin kinase eluted by 1.5 M NaCl utilized both ATP and GTP as phosphoryl donors. In other experiments, when the column was eluted by a linear NaCl gradient, the major phosvitin kinase eluted at an ionic strength of about 0.8 (data not shown). In the experiment depicted in Fig. 3, no detectable glycogen synthase kinase activity eluted with phosvitin kinase in the 1.5 M wash. Since the carry over of NaCl in the glycogen synthase assay was about 300 mM, it was possible that enzyme activity might be inhibited by salt [17]. Therefore, fractions corresponding to the peak of phosvitin kinase activity were desalted by gel filtration on Sephadex G-50 using the rapid centrifugation method of Neal and Florini [36]. However, even after desalting, no glycogen synthase kinase activity was detected. In another experiment where the phosvitin kinase was more concentrated, approx. 3% of the total glycogen synthase kinase activity eluted from the column was represented by this fraction [16]. This procedure, therefore, separated the major glycogen synthase kinase fraction from phosphorylase kinase, essentially all of the phosvitin kinase and the holoenzyme of cyclic AMP-dependent protein kinase.

The fractions in the 0.5 M eluate which contained glycogen synthase kinase activity were pooled and potassium phosphate was added to give a final concentration of 100 mM at pH 7.0. The enzyme was then precipitated by the addition of 0.48 g $(NH_4)_2SO_4$/ml. After stirring for 1 h, the suspension was centrifuged at 30 000 × *g* for 15 min. The pellet was taken up in about 2 ml of 50 mM K_2HPO_4/50 mM KH_2PO_4/200 mM NaCl/1 mM EDTA (buffer D) and dialyzed twice against 500 ml buffer D for 2 h each. A 2 ml aliquot of the dialyzed sample was applied to a Sephadex G-100 column (1.5 × 84 cm) which had been equilibrated with buffer D and calibrated with protein standards. This step resolved glycogen synthase kinase into three peaks designated I, II, and III with apparent molecular weights of 68 000, 52 000 and 39 000, respectively (Fig. 4). Both peak I kinase and peak II kinase phosphorylated as well as inactivated glycogen synthase. For both kinases, inactivation was ATP-dependent indicating that the inactivation was due to phosphorylation and not proteolysis. The glycogen synthase kinase activity of peaks I and II was insensitive to the heat-stable inhibitor of cyclic AMP-dependent protein kinase, whereas the glycogen synthase kinase and the histone kinase activity of peak III were inhibited by the inhibitor. Therefore, peak III is probably the free catalytic subunit of the cyclic AMP-dependent protein kinase, which is known to have a molecular weight of 41 000 [27,37]. Further support for this conclu-

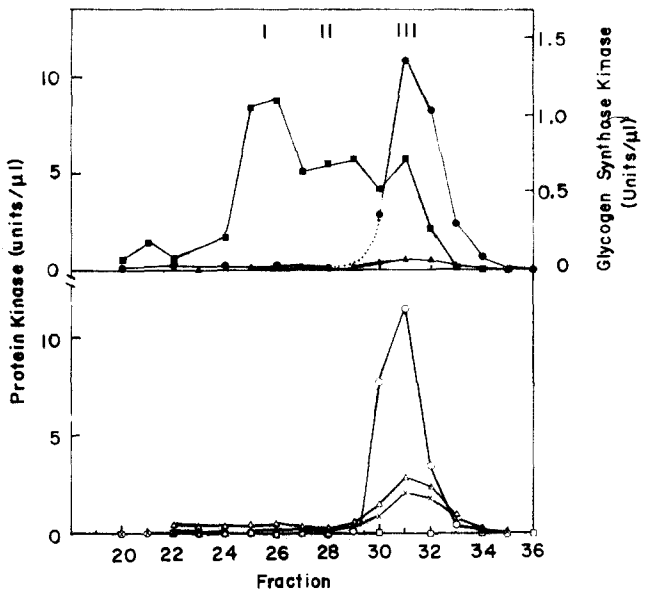


Fig. 4. Sephadex G-100 chromatography of glycogen synthase kinase. 2 ml of glycogen synthase kinase purified through the $(NH_4)_2SO_4$ and dialysis step were chromatographed. Fractions (2 ml) were assayed for inactivation of glycogen synthase (■———■) and for phosphorylation of histone (●———●), phosvitin (▲———▲), casein (X———X), protamine (△———△), phosphorylase (□———□), or phosphorylase kinase (○———○) as described previously [16] with 2(*N*-morpholine) ethansulfonate buffer at pH 6.0 for phosvitin and casein, and pH 6.5 for the other substrates. Protein substrate concentrations were 6 mg/ml except for phosphorylase and phosphorylase kinase which were at 0.2 mg/ml. Fractions 25 and 26 were pooled for peak I and fraction 28 and 29 were pooled for peak II.

sion comes from the fact that this fraction also catalyzed the phosphorylation of phosphorylase kinase, protamine, and casein. In contrast peaks I and II showed little or no phosphorylation of the other protein substrates. No phosphorylase kinase activity was detectable in any of the fractions. Since there were interfering substances in the extract, it was not possible to accurately determine the degree of purification or recovery of glycogen synthase kinase activity. However, this scheme removed cyclic AMP-dependent protein kinase, its catalytic subunit, phosphorylase kinase and the major phosvitin kinase from the glycogen synthase kinase. Pooled fractions of peak I or II could be stored at 4°C for several months with only small losses in activity.

The relative amounts of peak I and peak II glycogen synthase kinase varied in different preparations. In addition, the peak I enzyme appeared to be diminished after the $(NH_4)_2SO_4$-precipitated fraction was stored for several days at 4°C before Sephadex G-100 chromatography. For example, in one such preparation, there were 3851 units of glycogen synthase kinase activity in the peak II pool and only 706 units of activity in the peak I pool. Thus, it was possible to prepare peak II glycogen synthase kinase that was virtually free of peak I enzyme. The studies reported in this paper used peak II glycogen synthase kinase unless otherwise indicated. Prior to some experiments the enzyme was concentrated in a dialysis bag surrounded by Sephadex G-200 or by collodium bag vacuum dialysis.

TABLE I

METAL ION AND TRINUCLEOTIDE SPECIFICITY OF GLYCOGEN SYNTHASE KINASE

Glycogen synthase kinase activity was determined by measuring the inactivation of glycogen synthase. The reaction mixture contained 1 mM trinucleotide, 3 mM metal ion and 0.9 mM EDTA.

Metal ion	Trinucleotide	Glycogen synthase kinase activity (units/ml reaction mixture)
Mg^{2+}	ATP	24
Mn^{2+}	ATP	5
Fe^{2+}	ATP	1
Ca^{2+}	ATP	0
Mg^{2+}	2'dATP	17
Mg^{2+}	GTP	4
Mg^{2+}	ITP	2
Mg^{2+}	CTP	2
Mg^{2+}	XTP	0
Mg^{2+}	UTP	0

Metal ion and nucleotide specificity

Glycogen synthase kinase activity was not affected by the addition of 0.2 mM Ca^{2+}, 1 mM EGTA, 5 μM cyclic AMP, 5 μM cyclic CMP, 5 μM cyclic GMP, 5 μM cyclic IMP, or 5 μM cyclic TMP (data not shown). In the presence of 1 mM ATP, the metal ion requirement could be met by either Mg^{2+} or Mn^{2+}, but not by Fe^{2+} or Ca^{2+} (Table I). Mg^{2+} was 5-times more effective than Mn^{2+}. Co^{2+} and Zn^{2+} had inhibitory effects on glycogen synthase activity and could not be included in this comparison. When the kinase was assayed with $MgCl_2$, the preferred substrate was ATP, although dATP was also effective (Table I). GTP, ITP, and CTP were relatively poor substrates and no activity was seen with UTP or XTP (Table I). The K_m ± S.E. for ATP was determined to be 16 ± 1 μM by fitting the data directly to the Michaelis-Menten equation by non-linear least-squares analysis [50]. The kinetics did not appear to be cooperative.

Phosphorylation and inactivation of glycogen synthase by the catalytic subunit of the cyclic AMP-dependent protein kinase, phosphorylase kinase, and glycogen synthase kinase

Phosphorylation and inactivation of glycogen synthase by several concentrations of catalytic subunit is shown in Fig. 5. In the control reaction where glycogen synthase was incubated in the absence of added kinase, there was very little phosphorylation or change in activity ratio. At a low concentration of catalytic subunit (0.85 units/μl) there was a rapid incorporation of 1 mol phosphate per mol of glycogen synthase subunit but no further detectable phosphorylation. There was only a small decrease in activity ratio associated with the first mol of phosphate added. When higher concentrations of catalytic subunit were employed more phosphate was incorporated and the activity ratio was decreased. The rate of phosphorylation decreased dramatically as the extent of phosphorylation increased. For example, taking into account both incubation time and kinase concentration, it can be estimated that the rate of phosphorylation of the first mol of phosphate added was approx. 30-times greater than that of the second mol added. When the concentration of catalytic

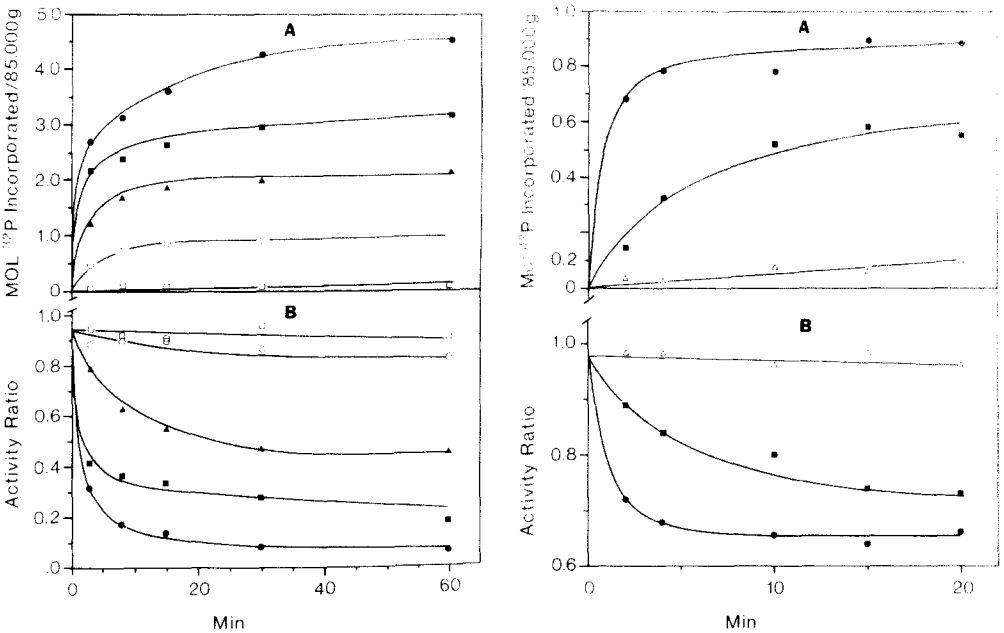


Fig. 5. Phosphorylation and inactivation of glycogen synthase by catalytic subunit of the cyclic AMP-dependent protein kinase. Catalytic subunit was added to yield a final concentration of 0 (□——□), 0.85 (○——○), 8.5 (▲——▲), 85 (■——■), or 340 (●——●) histone units/μl. At the indicated times, aliquots were removed for measurement of ^{32}P incorporation into glycogen synthase (Panel A) or glycogen synthase activity ratio (Panel B).

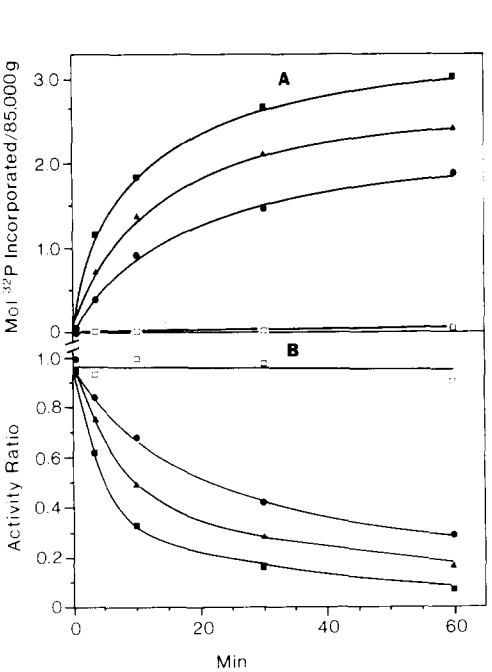
Fig. 6. Phosphorylation and inactivation of glycogen synthase by phosphorylase kinase. The concentration of ATP was 0.9 mM and in some cases EGTA was replaced by 0.2 mM $CaCl_2$ + 10 μg Ca^{2+}-dependent regulator protein/ml. The reaction mixtures contained either EGTA and no added kinase (△——△, Ca^{2+}, Ca^{2+}-dependent regulator protein and no added kinase (■——■) or Ca^{2+}, Ca^{2+}-dependent regulator protein + 3.2 μg phosphorylase kinase/ml (●——●). The phosphorylase kinase had been activated by preincubation with 1.6 mM ATP and 16 mM Mg^{2+} [21]. At the indicated times aliquots were removed for measurement of ^{32}P incorporation into glycogen synthase (Panel A) or glycogen synthase activity ratio (Panel B).

subunit tested was 8.5 units/μl about 2 mol phosphate per mol subunit were incorporated and the activity ratio was reduced to about 0.5 (Fig. 5). This degree of inactivation could occur in vivo since this concentration of kinase is only 33% of the total cyclic AMP-dependent protein kinase found in rat skeletal muscle [16]. At higher concentrations, 3—4 mol phosphate per mol subunit could be incorporated and the activity ratio was reduced to about 0.1 (Fig. 5). Since greater than physiological concentration of cyclic AMP-dependent protein kinase was required to decrease the activity ratio below 0.3, the low activity ratios which can be found in vivo [1] probably result in part from the activity of other kinases.

Phosphorylation and inactivation of glycogen synthase by phosphorylase kinase is shown in Fig. 6. In the presence of EGTA, there was only a small amount of phosphorylation and a slight inactivation. This glycogen synthase preparation contained calcium-dependent phosphorylase kinase [21], which phosphorylated and inactivated glycogen synthase when Ca^{2+} and Ca^{2+}-dependent regulator protein were present. The maximum amount of glycogen syn-

thase phosphorylation catalyzed by added phosphorylase kinase was about 0.9 mol per mol subunit and the activity ratio was reduced to 0.6. Similar results were obtained when phosphorylase kinase was added to a glycogen synthase preparation which was free of contaminating phosphorylase kinase (data not shown).

The time course of glycogen synthase phosphorylation and inactivation catalyzed by several concentrations of peak II glycogen synthase kinase is shown in Fig. 7. There was a marked decrease in the activity ratio associated with the first mol of phosphate incorporated and with subsequent phosphorylation. The activity ratio was reduced to about 0.1 with the addition of three phosphates per subunit. While there was a decrease in the rate of phosphorylation of each successive mol, incorporation of each additional mol required an increase in incubation time and/or enzyme concentration of only 2—3-fold. Thus, the reaction rate was not as sensitive to phosphorylation state as when the catalytic subunit was used (see above). Similar results were obtained with peak I glycogen synthase kinase (data not shown). At higher concentrations of the kinase, up to 4 mol per mol of subunit could be incorporated.



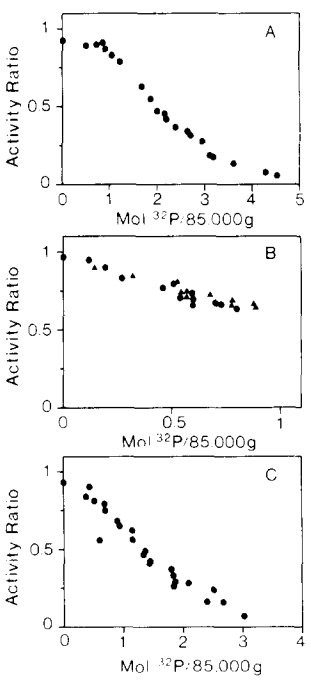


Fig. 7. Phosphorylation and inactivation of glycogen synthase by glycogen synthase kinase. Glycogen synthase kinase was added to yield a final concentration of 0 (□———□), 0.06 (●———●), 0.13 (▲———▲), 0r 0.26 (■———■) inactivation units/μl. At the indicated times, aliquots were removed for measurement of ^{32}P incorporation into glycogen synthase (Panel A) or glycogen synthase activity ratio (Panel B).

Fig. 8. Correlation of glycogen synthase activity ratio with phosphorylation catalyzed by catalytic subunit (Panel A), by phosphorylase kinase (Panel B) or by glycogen synthase kinase (Panel C). The activity ratio was plotted as a function of the phosphorylation state using data obtained from experiments such as those described in Fig. 5—7. The phosphorylase kinase reaction was carried out in the absence (●———●) and the presence (▲———▲) of 10 μg Ca^{2+}-dependent regulator protein/ml.

The correlation of phosphorylation and activity ratio for the three kinases is presented in Fig. 8. Of the three kinases glycogen synthase kinase is clearly the most effective for inactivation of glycogen synthase under these conditions. With phosphorylase kinase there was a linear relationship between inactivation and phosphorylation up to 1 mol per mol of glycogen synthase subunit. The results with phosphorylase kinase are similar to the results of DePaoli-Roach et al. [18] but in contrast to the results of Soderling et al. [38]. The latter found that glycogen synthase inactivation was correlated with only the first 0.5–0.6 mol phosphate per mol subunit and subsequent phosphorylation was not associated with activity ratio changes. The addition of Ca^{2+}-dependent regulator protein to the phosphorylase kinase catalyzed reaction did not change the relationship between phosphorylation and inactivation (Fig. 8). Thus, Ca^{2+}-dependent regulator protein stimulated the rate of phosphorylation but did not change the specificity of phosphorylase kinase.

Phosphorylation of specific regions in glycogen synthase

Phosphorylation regions of glycogen synthase can be designated as trypsin-sensitive and trypsin-insensitive regions based on solubility (sensitive) or insolubility (insensitive) in trichloroacetic acid of the peptides which result from mild trypsin treatment [39]. [^{32}P]Glycogen synthase which had been phosphorylated in the presence of catalytic subunit, glycogen synthase kinase, or phosphorylase kinase was treated with trypsin [39] and separated into trichloroacetic acid-soluble and trichloroacetic acid-insoluble peptides. Trypsin solubilized 95% of the first phosphate introduced by the catalytic subunit but only 17% of the first phosphate introduced by glycogen synthase kinase (Table II). Thus, for the first mol of phosphate incorporated, the catalytic subunit is highly specific for a site(s) which is trypsin-sensitive and does not change the activity ratio whereas glycogen synthase kinase is specific for a site(s) which is trypsin-insensitive and markedly reduces the activity ratio (Table II, Fig. 8). Further phosphorylation by either kinase resulted in phosphorylation of both trypsin-sensitive and trypsin-insensitive sites. When phosphorylase kinase was

TABLE II

DISTRIBUTION OF PHOSPHATE BETWEEN TRYPSIN-SENSITIVE AND TRYPSIN-INSENSITIVE REGIONS

[^{32}P]Glycogen synthase was prepared to various degrees of phosphorylation with the indicated kinase. Trichloroacetic acid-precipitable [^{32}P]protein was determined as described by Reimann et al. [34], before and after mild trypsin treatment as described by Soderling [39]. The phosphate precipitated after trypsin treatment was defined as trypsin-insensitive phosphate. The difference between total phosphate (before trypsin treatment) and trypsin-insensitive phosphate was defined as trypsin-sensitive phosphate [39].

Kinase	Phosphates/subunit		
	Trypsin-sensitive	Trypsin-insensitive	Total
Catalytic subunit	0.80	0.04	0.84
Catalytic subunit	1.40	0.50	1.90
Catalytic subunit	2.33	1.62	3.95
Glycogen synthase kinase	0.15	0.70	0.85
Glycogen synthase kinase	1.64	2.07	3.71
Phosphorylase kinase	0.12	0.35	0.47

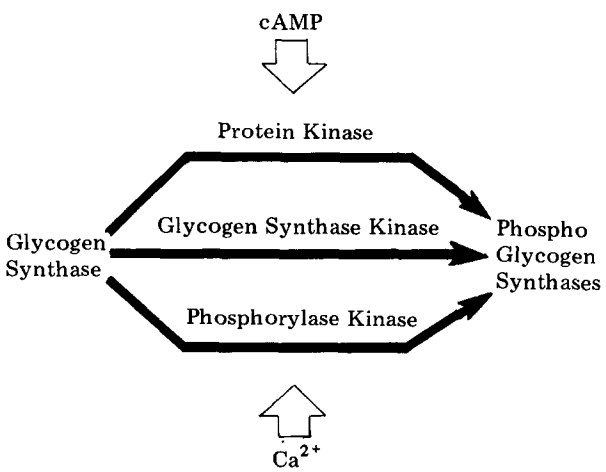
used to phosphorylate glycogen synthase to about 0.5 mol/mol subunit, the phosphate was predominantly trypsin-insensitive and there was a decrease in activity ratio (Table II, Fig. 8).

Effect of glycogen on glycogen synthase phosphorylation

Glycogen regulates the activity of glycogen synthase by a feedback mechanism involving inhibition of glycogen synthase phosphatase [40], thereby favoring the less active, phosphorylated forms of glycogen synthase. It seemed possible that a complementary mechanism involving kinase activation might occur. Soderling [41] has shown that glycogen has no effect on the rate at which cyclic AMP-dependent protein kinase phosphorylates glycogen synthase. Using the catalytic subunit we also found no effect of glycogen (results not shown). The activity of glycogen synthase kinase was enhanced only about 25% by glycogen (results not shown). Thus, glycogen does not appear to be an important regulator of either kinase. Glycogen levels are also presumably regulated by an allosteric mechanism involving stimulation of phosphorylase kinase by glycogen causing increased glycogenolysis [42]. In agreement with Krebs et al. [42], we found that glycogen increased phosphorylase activity 8-fold when the substrate was phosphorylase *b* [43]. However, the rate at which phosphorylase kinase phosphorylated glycogen synthase was enhanced only about 25% by glycogen (results not shown). In similar experiments DePaoli-Roach et al. [20] found that glycogen increased the rate of phosphorylation of glycogen synthase by 13%. Thus, it seems unlikely that the rate of glycogen synthase phosphorylation is significantly enhanced by glycogen, although increased sensitivity to glycogen might occur in the presence of other effectors found in vivo.

Discussion

Skeletal muscle glycogen synthase can be phosphorylated and inactivated by three types of protein kinase (Scheme I). There is evidence that glycogen syn-



Scheme I. Phosphorylation of glycogen synthase by three types of protein kinase.

thase activity can be regulated by cyclic AMP via changes in cyclic AMP-dependent protein kinase activity [4]. Similarly, it seems likely that calcium might also play a regulatory role via changes in phosphorylase kinase activity [19—23] although direct evidence for this mechanism in skeletal muscle has not been obtained. There is evidence that calcium may be required for phosphorylation and inactivation of glycogen synthase by hormones in isolated adipocytes [44] and hepatocytes [45]. In contrast, the effects of insulin on glycogen synthase activity in skeletal muscle are not related to changes in cyclic AMP concentration [46] and do not require the presence of phosphorylase kinase [47]. Under such conditions, factors other than calcium or cyclic AMP presumably regulate glycogen synthase phosphatase and/or glycogen synthase kinase. Because of the high concentrations of glycogen synthase kinase in many tissues [16], and its presumed role in glycogen metabolism it is important to characterize and to establish how the enzyme is regulated.

The procedure described in this study resulted in a preparation of two rat skeletal muscle glycogen synthase kinases, one designated peak I with an apparent molecular weight of 68 000 and the other designated peak II with an apparent molecular weight of 52 000. Both kinases were essentially free of cyclic AMP-dependent protein kinase and phosphorylase kinase. In addition, the procedure separated glycogen synthase kinase from the major phosvitin kinase which was eluted from the phosphocellulose column at high ionic strength. Phosvitin kinase utilized both ATP and GTP as phosphoryl donors and appeared to be similar to the kinase purified from rabbit skeletal muscle by other investigators [10,13,18]. Since glycogen synthase kinase activity was not associated with any other major protein kinase activity tested, successful purification of this enzyme will probably require the use of glycogen synthase as the substrate to measure glycogen synthase kinase activity throughout a purification procedure. Using the inactivation assay, we were able to assay glycogen synthase kinase in tissue extracts even in the presence of interfering substances, whereas excessive phosphorylation of endogenous protein in the extracts made it difficult to measure phosphorylation of glycogen synthase.

Larner and coworkers [18] recently classified glycogen synthase kinases isolated from rabbit skeletal muscle into two groups: those which elute from phosphocellulose at high ionic strength and appear to be associated with phosvitin kinase and those which elute at low ionic strength. Peak I and II glycogen synthase kinase from rat skeletal muscle would fit into the latter group and share some but not all of the properties reported for enzyme(s) of this group. In agreement with previous reports, GTP was a relatively poor phosphate donor [18], and the K_m for ATP was less than 20 μM [17]. The most striking difference was the apparent molecular weight determined by gel filtration. Itarte and Huang [17] reported a molecular weight of approx. 30 000 compared to 68 000 and 52 000 found in this study. There were also differences in the relationship between phosphorylation and inactivation of glycogen synthase (see below).

A great deal of variation has been reported in both the extent of phosphorylation and the relationship between phsophorylation and inactivation of glycogen synthase catalyzed by well characterized preparations of cyclic AMP-dependent protein kinase [5,7,10,13,15,18,24,42,48,49]. These variations include

1 mol phosphate per mol subunit having little effect on activity ratio, 1 mol phosphate per mol subunit producing near complete inactivation (activity ratio less than 0.2), and 3—4 phosphates per subunit required to inactivate glycogen synthase. These differences presumably are due to variations both in the glycogen synthase used as substrate and in the glycogen synthase kinase reaction conditions. For example, glycogen had a small but definite effect on the activity of glycogen synthase kinase. In this study we used fixed conditions to compare the patterns of phosphorylation and inactivation of glycogen synthase when catalyzed by the catalytic subunit of the cyclic AMP-dependent protein kinase, by phosphorylase kinase and by glycogen synthase kinase. As we [5,24] and DePaoli-Roach et al. [18] had previously reported, incorporation of up to 1 mol phosphate per mol subunit catalyzed by the cyclic AMP-dependent protein kinase had very little effect on the activity ratio. Nearly all of this phosphate was trypsin-sensitive, i.e., rendered trichloroacetic acid soluble by mild tryptic hydrolysis. In another study [5], we showed that the first mol phosphate incorporated was kinetically 'silent', i.e., it did not change the kinetics for the substrate UDPGlc or the activator glucose-6-*P*. Incorporation of approx. 3 mol phosphate per mol subunit was required to reduce the activity ratio to 0.1. Phosphorylase kinase apparently catalyzes incorporation of only about 1 mol phosphate per mol synthase subunit resulting in a decrease in activity ratio from 0.97 to 0.6 (Figs. 6 and 8). Others have reported that phosphorylation is restricted to a unique serine residue, seven amino acids from the N-terminus [23,38]. Although we have not done sequence studies, our trypsin experiments are consistent with most of the phosphorylation occurring at this site. Purified peak II glycogen synthase kinase catalyzed the incorporation of 3—4 mol per mol subunit. In contrast to cyclic AMP-dependent protein kinase, the first mol phosphate per mol subunit was primarily trypsin-insensitive and there was a decrease in activity ratio associated with each of the first three phosphates incorporated per subunit. After the addition of 3 mol phosphate per subunit, the activity ratio was about 0.1.

The regulation of glycogen synthase activity is very complex. It is clear that each of these three types of protein kinase have different specificities for phosphorylation sites on glycogen synthase and can produce a number of distinctly different molecular forms of glycogen synthase. In vivo, these kinases acting individually or in combination could lead to a large number of phosphorylated forms of glycogen synthase with different kinetic properties providing for intricate regulation of glycogen synthase activity.

Acknowledgments

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