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Conversion of Skeletal Muscle Glycogen Synthase to Multiple Glucose 6-Phosphate Dependent Forms by Cyclic Adenosine Monophosphate Dependent and Independent Protein Kinases†

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ABSTRACT: Glycogen synthase was purified from rabbit skeletal muscle with a glucosamine 6-phosphate affinity column as the final step of purification. The product was primarily a trimer with a molecular weight of 269 000, contained less than 0.1 mol of alkali-labile phosphate/10^5 g of enzyme, had a −:+ glucose 6-phosphate (Glc-6-P) activity ratio of 0.7, and demonstrated positive cooperativity with respect to Glc-6-P. This activator produced a tenfold increase in the affinity of the nonphosphorylated enzyme for uridine diphosphoglucose with an $A_{0.5}$ for Glc-6-P of 15-25 μM. Phosphorylation of the purified glycogen synthase with the catalytic subunit of cAMP-dependent protein kinase resulted in incorporation of up to 2 mol of phosphate/10^5 g and increased the $A_{0.5}$ for Glc-6-P to ~250 μM. Incorporation of 1 mol of phosphate/10^5 g could also be achieved using a cAMP-independent synthase kinase purified from rabbit skeletal muscle. Glycogen synthase phosphorylated with the synthase kinase had a lower activity ratio and a higher $A_{0.5}$ for Glc-6-P (~500 μM) than glycogen synthase phosphorylated by the cAMP-dependent protein kinase. When glycogen synthase was phosphorylated with both the cAMP-dependent and cAMP-independent kinases, 3 mol of phosphate/10^5 g was incorporated, suggesting the existence of at least three distinct sites of phosphorylation. The $A_{0.5}$ for Glc-6-P increased progressively with phosphorylation to 3 mol/10^5 g ($A_{0.5} \sim 1800$ μM), while the −:+ Glc-6-P activity ratio reached a minimal value (0.02) after incorporation of less than 2 mol/10^5 g. These results indicate that the apparent affinity of muscle glycogen synthase for Glc-6-P is a more sensitive and accurate indicator of regulation of the enzyme by phosphorylation than is the −:+ Glc-6-P activity ratio. Phosphorylation of the synthase by a cAMP-independent protein kinase in combination with the cAMP-dependent protein kinase yields a Glc-6-P-dependent form of glycogen synthase that would probably be completely inactive at physiological concentrations of Glc-6-P.

Glycogen synthase from skeletal muscle is converted during solubilization and purification to a form that contains little alkali-labile phosphate and that is active in the absence of added Glc-6-P (Soderling et al., 1970; Roach et al., 1976). This Glc-6-P independent form of the enzyme ("I" form) can be phosphorylated in the presence of ATP and Mg^{2+} to a form that is dependent on Glc-6-P ("D" form) for activity (Friedman and Larner, 1963).

Conversion of glycogen synthase from the I to the D form has generally been determined by measuring decreases in the −:+ Glc-6-P activity ratio. Phosphorylation of glycogen synthase with cAMP[1]-dependent protein kinase results in a de-

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[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; BSA, crystalline bovine serum albumin; Tes, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Glc-6-P, glucose 6-phosphate; CM, carboxymethyl; DEAE, diethylaminoethyl; NADH, nicotinamide adenine dinucleotide reduced; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

crease in this ratio (Soderling et al., 1970; Villar-Palasi et al., 1971; Soderling, 1975) as does phosphorylation with cAMP-independent glycogen synthase kinase[2] (Nimmo et al., 1976b; Itarte et al., 1977). Glycogen synthase from skeletal muscle has been reported to be completely converted to the dependent form by phosphorylation to 2 mol of phosphate per subunit with the catalytic subunit of cAMP-dependent protein kinase (Soderling, 1975), with a cAMP-independent synthase kinase (Itarte et al., 1977), or with a combination of cAMP-dependent protein kinase and cAMP-independent synthase kinase (Huang et al., 1975; Nimmo et al., 1976b).

Phosphorylation of glycogen synthase results not only in a decreased −:+ Glc-6-P activity ratio, but also in a lower apparent affinity of the enzyme for this activator (Roach et al., 1976; Nimmo et al., 1976). Changes in the activation constant for Glc-6-P associated with phosphorylation provide a further index of conversion of glycogen synthase to dependent forms. We have examined alterations in the kinetic properties of glycogen synthase phosphorylated with the catalytic subunit of cAMP-dependent protein kinase, with a cAMP-independent glycogen synthase kinase and with a combination of these kinases in order to compare more precisely the control of glycogen synthase activity with the various patterns of phosphorylation.

Experimental Procedures

Methods

Glycogen Synthase Purification and Assay. Glycogen synthase was purified from fresh rabbit skeletal muscle by modifications of the method of Soderling et al. (1970). The resuspended glycogen pellet (30P) produced by centrifugation at 30 000 rpm in a Beckman Type 30 rotor was further purified without freezing, since freezing resulted in a 60% loss of glycogen synthase activity. Considerable loss of glycogen synthase activity (up to 60%) also occurred when the resuspended 30P was washed and centrifuged again. The glycogen pellet (30P) was resuspended as described by Soderling et al. (1970) except that, in preparation 3, phenylmethylsulfonyl fluoride (2 mM), ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA, 2 mM), and glycerol (10%) were also added. Purified human salivary amylase (Shainkin and Birk, 1966) was added to the resuspended glycogen pellet at a concentration of 40 μg/mL, and the preparations were incubated for 1–2 h at 30 °C. The concentration of amylase was increased approximately tenfold in preparations 2b and 3 to achieve more complete solubilization of the enzyme; preparation 3 was incubated overnight at room temperature. Following centrifugation at 30 000 rpm for 30 min, the supernatant fraction was applied to a Whatman DE-52 column, washed, and eluted as described (Soderling et al., 1970). The pooled DE-52 fractions were stirred for 2 h at 4 °C with 1–2 volumes of glucosamine 6-phosphate–Sepharose 4B prepared according to the method of Miller et al. (1975) (2.6–3.6 μmol/mL wet packed gel). A column (2 × 75 cm) of the mixture was then poured, washed with 1 bed volume of 500 mM Tris, 1 mM EDTA, 5% sucrose, 40 mM 2-mercaptoethanol, pH 7.5, and then the Tris concentration was reduced to 150 mM (1 bed volume). Glycogen synthase was eluted from the affinity column with 50 mM sodium sulfate in the same buffer but containing 50 mM Tris, pH 7.8. The fractions containing glycogen synthase activity were pooled and concentrated in dialysis tubing surrounded by Ficoll (Pharmacia). The concentrated enzyme was then dialyzed and glycerol added to a final concentration of 25%. Recovery of glycogen synthase activity was from 3.5 to 7.5% for the various preparations. Samples were stored in 100–200-μL aliquots at −70 °C and were stable for at least 6 months. Additional variations in the methods of purification are described in the footnotes to Table I.

Glycogen content of the final product of purification was determined by enzymatic degradation with glycogen phosphorylase a and debranching enzyme coupled to NADH production with phosphoglucomutase and Glc-6-P dehydrogenase (Lowry and Passonneau, 1972).

Glycogen synthase activity was assayed by the method of Thomas et al. (1968). Incubations were carried out for 20 min at 30 °C in a reaction volume of 125 μL with UDP-[^{14}C]-glucose as substrate. For determination of total glycogen synthase activity and activity ratios (minus Glc-6-P:7.5 mM Glc-6-P), UDP-[^{14}C]glucose was present at a final concentration of 4.6 mM (0.15 μCi/μmol). For determination of the $A_{0.5}$ for Glc-6-P, UDP-[^{14}C]glucose was present at a final concentration of 50 μM (10.5 μCi/μmol). Glycogen synthase was diluted in 50 mM Tris, pH 7.8, 1 mM EDTA, 20 mM KF, 0.25 M sucrose, and 0.1% crystalline bovine serum albumin (BSA) from 200- to 6000-fold as required to keep substrate consumption below 10% in the assay. The reaction mixture also contained 25 mM Tris buffer (pH 7.8), 1 mM EDTA, and 8 mg/mL oyster glycogen purified by a modification of the method of Somogyi (1957). Reactions were initiated by the addition of 50 μL of enzyme and terminated by spotting 100 μL of the reaction mixture onto Whatman 3MM paper disks which were then washed twice in 66% ethanol (45 and 15 min), dehydrated in 95% ethanol, and counted by liquid scintillation spectrometry in toluene containing Omnifluor (4 g/L).

Glycogen Synthase Phosphorylation. Phosphorylation reactions were carried out at 30 °C in a final volume of 50–300 μL. The reaction mixture contained purified glycogen synthase I (0.4–1 mg/mL), 25 mM Tes buffer, pH 7.0, 10 mM magnesium acetate, 100 mM 2-mercaptoethanol, [γ-^{32}P]ATP (2–5 × 10^8 cpm/μmol) or unlabeled ATP, and cAMP-dependent protein kinase, cAMP-independent synthase kinase, or both kinases. The concentration of ATP in the reaction mixture was 0.2 mM when partially purified cAMP-dependent protein kinase or the catalytic subunit of cAMP-dependent protein kinase was used and 1.0 mM when synthase kinase or both kinases were used. cAMP (10 μM) was included only when partially purified cAMP-dependent protein kinase was used. Reactions were initiated by the addition of ATP. For determination of protein-bound phosphate, aliquots of the reaction mixture were removed at various times, diluted in 300 μL of ice-cold 0.15% BSA, and 100 μL of this solution was plated in triplicate onto Whatman 3MM paper disks which were washed three times (30 min each) in cold 10% Cl_3AcOH, dehydrated in 95% ethanol, dried, and counted by liquid scintillation spectrometry in toluene containing Omnifluor (4 g/L). Aliquots removed for assay of glycogen synthase were diluted 50- to 1000-fold prior to assay. Only cold ATP was used in the phosphorylation mixture when glycogen synthase activity was to be assayed, since ^{32}P interfered with the measurement of UDP-[^{14}C]glucose incorporation into glycogen. Comparisons of phosphate content and glycogen synthase activity were therefore made on separate phosphorylation mixtures assayed simultaneously, or in separate experiments run under identical conditions.

[2] The term "glycogen synthase kinase" is used here, as by others (Schlender and Reimann, 1975; Nimmo et al., 1976b; Itarte et al., 1977), to denote the cAMP-independent kinase activity that phosphorylates glycogen synthase. The enzyme used in our studies was purified on the basis of activity relative to casein and may not represent all of the synthase kinase activity in skeletal muscle.

[γ-^{32}P]ATP was prepared as described previously (Mayer et al., 1974) or was obtained commercially. ATP was measured fluorometrically (Lowry and Passonneau, 1972). The contamination with $^{32}P_i$ in the labeled ATP preparations was determined by adsorption of ATP onto charcoal, and was <5% for all preparations of [^{32}P]ATP used.

Purification and Assay of cAMP-Dependent Protein Kinase. cAMP-dependent protein kinase was purified approximately 100-fold from rabbit skeletal muscle by a modification of the method of Walsh (Wastila et al., 1971). The preparations used had specific activities of approximately 0.14 nmol min^{-1} mg of protein^{-1} when assayed using casein dephosphorylated by the method of Reimann et al. (1971) as substrate.

The catalytic subunit of cAMP-dependent protein kinase was prepared from 2.5–3 kg of freshly excised rabbit muscle by a modification of the method of Beavo et al. (1974) (de Maille et al., unpublished). The catalytic subunit eluted from CM-Sephadex between 100 and 200 mM potassium phosphate was dialyzed against 5 mM potassium phosphate, 5 mM magnesium acetate, 15 mM 2-mercaptoethanol, pH 7.0, and applied to a column (20-mL bed volume) of Sepharose 4B coupled to Cibacron blau F3GA (Ryan and Vestling, 1974) which was washed with 5 bed volumes of dialyzing buffer. The enzyme was eluted with 3–4 bed volumes of dialyzing buffer containing 0.4 N NaCl. Acrylamide gel thin-layer electrophoresis showed the enzyme migrating as a single band. Activity of the catalytic subunit was assayed in a final volume of 70 μL containing 20 μL of enzyme (diluted in 30 mM potassium phosphate, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mg/mL BSA, pH 7.0) and 50 μL of a solution containing 50 mM potassium phosphate, pH 7.0, 30 mg/mL histone IIA, 18 mM magnesium acetate, and 1 mM [γ-^{32}P]ATP (5×10^8 cpm/μmol). After incubation for 20 min at 30 °C, 50-μL aliquots were spotted onto paper disks which were washed three times (30 min each) in 10% Cl_3AcOH, dehydrated, dried, and counted, as described above.

Purification and Assay of cAMP-Independent Protein Kinase (Glycogen Synthase Kinase). Two kilograms of freshly excised rabbit back and leg muscle were ground and then homogenized in a Waring blender with 2.5 volumes of an aqueous solution of 4 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.0. The supernatant extract after centrifugation was adjusted to pH 6.1 with 1 N acetic acid and again centrifuged. The clear supernatant solution was adjusted to a conductivity of 0.8 mmhos with a solution of 10 mM potassium phosphate, 15 mM 2-mercaptoethanol, pH 6.8 (buffer A). DE-52 cellulose was equilibrated with this solution and 1.2 L of the packed cellulose mixed with the muscle extract for 15 min. The cellulose was poured onto a sintered glass funnel and washed with buffer A. Then the cellulose was poured into an 8-cm diameter column (total volume about 4 L) and washed with 10 bed volumes of buffer A but containing 50 mM phosphate until the nonadsorbed proteins had been removed. cAMP-dependent protein kinase catalytic subunit was removed by 10 μM cAMP in buffer A (10 L at 1 L/h). A linear NaCl gradient, 0 to 0.5 M NaCl, in buffer A was started with 2 L in each gradient tube at a rate of 1000 mL/h. Three peaks of cAMP-independent casein kinase activity were eluted at conductivity values of 17, 20, 24 mmhos. Each fraction was brought to 55% saturation with ammonium sulfate. The precipitates were redissolved in buffer A and dialyzed against three changes of 20 L of buffer A for 16 h total. The three dialyzed fractions remained turbid even after centrifugation for 1 h at 100 000g. Each fraction was adjusted to pH 6.5 with 1 M H_3PO_4 and mixed with 50 mL of packed phosphocellulose. The phosphocellulose had been washed sequentially with 1 N NaOH, H_2O, 1 N HCl, H_2O, and then equilibrated with buffer A at pH 6.5. Thorough washing of the phosphocellulose was essential for reproducible adsorption and elution of casein kinase activity. This activity was eluted from the phosphocellulose with a 0.5 to 1 M NaCl linear gradient in buffer A (100 mL in each gradient tube) at a rate of 75 to 100 mL/h. Activity eluted with each of the three fractions when conductivity was 55 to 60 mmhos. The three fractions were concentrated to <10 mL in Amicon cells and stored at 0 °C without removal of the NaCl. Each of the three preparations subjected to acrylamide gel electrophoresis in 7.5% $NaDodSO_4$ yielded the same two protein bands with estimated molecular weights of 92 000 and 82 000. Electrophoresis in 5% gels without $NaDodSO_4$ showed a single band migrating the same distance with each preparation.

cAMP-independent synthase kinase activity was assayed using casein, dephosphorylated as described by Reimann et al. (1971), as substrate at 7 mg/mL. The assay mixture also contained, in a final volume of 70 μL: 36 mM potassium phosphate, pH 6.8, 0.1 mM EGTA, 12 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (10^8 cpm/μmol), 1 mg/mL heat-stable inhibitor of protein kinase, and 20 μL of the purified enzyme. After incubation for 15 min at 30 °C, 50 μL was removed and plated onto 3MM paper disks which were washed three times for 30 min with 10% Cl_3AcOH, dried, and counted. The activity that eluted from DE-52 at 24 mmhos was used in the phosphorylation of glycogen synthase and had a specific activity of 1.4 nmol of phosphate incorporated into casein min^{-1} mg of protein^{-1}. Casein was the only substrate used to follow purification, and the substrate specificity of the final product of purification was not characterized.

The heat-stable inhibitor of cAMP-dependent protein kinase was purified approximately 1200-fold by a modification (de Maille et al., unpublished) of the method of Ashby and Walsh (1974), using a batch adsorption and elution from DE-52 followed by phosphocellulose column chromatography. One milligram of the purified product inhibited the incorporation of 2 nmol of phosphate/min into histone by the catalytic subunit.

Determination of Alkali-Labile Phosphate in Glycogen Synthase I. Duplicate samples of 200–225 μg of synthase protein were precipitated three times with 15% Cl_3AcOH and redissolved twice in 0.1 N NaOH. The third Cl_3AcOH precipitate was heated in 15% Cl_3AcOH at 90 °C for 20 min to dissolve any contaminating nucleic acids and was then washed with 5% Cl_3AcOH. It was then dissolved in 0.25 N NaOH and incubated at 37 °C for 16.5 h. The protein was again precipitated with 15% Cl_3AcOH, and both the supernatant and precipitated protein were washed separately (Ames, 1966) and analyzed for phosphate by the Malachite Green method (Itaya and Ui, 1966).

Materials

ATP (disodium salt), cAMP, Glc-6-P, UDP-glucose, oyster glycogen, histone IIA, and phosvitin were obtained from Sigma Chemical Co. Purified casein was obtained from Matheson, Coleman, and Bell. Sepharose 4B was from Pharmacia Fine Chemicals, phosphocellulose from Serva, carboxymethylcellulose from Sigma, and DEAE-cellulose (DE 52) from Whatman. UDP-[U-^{14}C]glucose (200 mCi/mmol) was from Amersham-Searle, and $^{32}P_i$ (carrier free) and [γ-^{32}P]ATP (10–30 Ci/mmol) were from ICN. Omnifluor was obtained from New England Nuclear Corp. Cibacron blau was a gift of Ciba-Geigy, Basel. Dialysis tubing (A. H. Thomas, Philadelphia) was cleaned by successive boiling in 1% acetic acid, 2% sodium bicarbonate with 1 mM EDTA, and 0.1 M sodium

TABLE I: Characterization of Purified Rabbit Muscle Glycogen Synthase I.[a]

Prep	Mol wt[b]	Sp act. (units/mg of protein)	Activity ratio −:+ Glc-6-P	Alkali-labile phosphate (mol/10^5 g of synthase)	$A_{0.5}$ Glc-6-P (μM)	n_H
2a		8.75	0.77	<0.1	11	1.3
2b		8.07	0.70 ± 0.01 (31)[d]	<0.1	21.2 ± 1.2[c]	1.53 ± 0.08[c]
$3a_1$	269 000	12.88	0.73 ± 0.02 (18)[d]	<0.1	15, 20	1.8, 2.0
$3a_2$	180 000–270 000	8.58	0.64 ± 0.01 (9)[d]	<0.1	17	1.6

[a] Glycogen synthase was purified from rabbit skeletal muscle as described under Experimental Procedures. Preparation 2 was divided into 2a and 2b after resuspension of the 30 000 rpm pellet (30P); preparation 2a was washed once while 2b was not. In preparation 3 the 30P was not frozen. Glycerol was added to the pooled DE-52 fractions in preparation 3 to stabilize the enzyme and to prevent aggregation. The enzyme was stirred with the glucosamine 6-phosphate affinity gel overnight. After the enzyme–affinity agarose column was poured, it was washed with the 500 mM Tris buffer described under Experimental Procedures and then with a 112 mM Tris buffer containing 25% glycerol (v/v). Preparation $3a_1$ was then eluted from the affinity column as described except that the buffer also contained 25% glycerol. Some enzyme activity ($3a_2$) was less tightly bound to the affinity resin, perhaps due to the inclusion of glycerol, and was eluted with the 112 mM Tris wash. [b] Molecular weight was determined by the meniscus depletion–equilibrium–sedimentation method (Yphantis, 1964). Each determination was made at two dilutions of the enzyme preparation which were centrifuged (at 12 000 rpm in a ANH-90 rotor in a Beckman Model E centrifuge) for 12 to 24 h. The partial specific volume was taken as 0.73 (Nimmo et al., 1976a). Calculations were based on interference fringe measurements. Preparation $3a_2$ was a heterogeneous mixture of molecules with molecular weights from 180 000 to 270 000. [c] Means ± standard errors of ten determinations. [d] Means ± standard errors; the number of determinations is in parentheses.

ascorbate, 1 mM EDTA. All solutions were prepared in glass-distilled deionized water or water purified through a Milli-Q system.

Results

Characterization of Purified Glycogen Synthase I. Several preparations of glycogen synthase, varying in some details of purification, were examined (Table I). $NaDodSO_4$ gel electrophoresis of glycogen synthase preparations 2a, $3a_1$, and $3a_2$ in 7.5% $NaDodSO_4$ showed identical patterns with one major band (molecular weight approximately 89 000) and a faint band of lower molecular weight. Molecular weight of the holoenzyme was calculated from equilibrium ultracentrifugation data (method of Yphantis, 1964). It was 268 000 ± 15 000 (standard error) for preparation $3a_1$. Preparation $3a_2$, which did not bind to the affinity column, was a mixture of dimer (180 000) and trimer (270 000) for which accurate molecular weights could not be calculated. The specific activity of the purified glycogen synthase preparations ranged from 8.1 to 12.9 μmol min^{-1} mg of $protein^{-1}$. The ratio of activity in the absence of Glc-6-P to that measured in the presence of 7.5 mM Glc-6-P was 0.64 for preparation $3a_2$ and 0.70–0.73 for the other preparations. Values for alkali-labile phosphate and for phosphate remaining in the Cl_3AcOH precipitate were <0.1 mol/10^5 g of glycogen synthase for all of the preparations, except 2a in which the value determined for the precipitate was 0.2 mol/10^5 g. The concentration of glycogen in the purified enzyme was <1%, w/w.

Analysis of the Glc-6-P activation kinetics of the purified enzyme revealed positive cooperativity for all preparations when assayed at a UDP-glucose concentration of 50 μM. A low substrate concentration was used because the stimulatory effect of Glc-6-P on activity of the I form of glycogen synthase is largely due to a decrease in the K_m of the enzyme for UDP-glucose. Since curves of velocity vs. Glc-6-P concentration were sigmoidal and therefore did not follow Michealis-Menten kinetics, the $A_{0.5}$ values for Glc-6-P were determined from Hill plots. The values ranged from 11 to 21 μM for all preparations, with Hill coefficients (n_H) between 1.3 and 2.0 (Table I). K_m values for UDP-glucose were determined for enzyme preparations 2b and $3a_1$. In the absence of Glc-6-P, the K_m values were 213 and 425 μM, respectively. In the presence of 7.5 mM Glc-6-P, the K_m values decreased to 51 and 25 μM, respectively.

In order to assess the degree of contamination of the purified glycogen synthase with protein kinase activity, phosphorylation of glycogen synthase was assayed under standard conditions but without the addition of exogenous protein kinase. Preparations 2b and $3a_1$ did not incorporate significant phosphate (<0.05 mol/10^5 g of glycogen synthase) after incubation with 1.0 mM ATP, in the presence or absence of cAMP, for up to 100 min. Under the same conditions, preparation $3a_2$ incorporated 0.2 mol of phosphate/10^5 g of glycogen synthase. This phosphorylation was seen even if cAMP was omitted, and reached maximal values between 30 and 60 min of incubation (data not shown).

Endogenous phosphatase activity was determined after phosphorylation of the purified glycogen synthase with added protein kinase and separation of the enzyme from [^{32}P]ATP on a Sephadex G-25 column. Subsequent incubation at 30 °C with 10 mM Mg^{2+} did not result in release of Cl_3AcOH-soluble ^{32}P from the phosphorylated glycogen synthase.

Phosphorylation of Glycogen Synthase I with cAMP-Dependent Protein Kinase. Several different preparations of glycogen synthase were incubated under standard phosphorylation conditions, with 0.2 mM ATP, 10 μM cAMP, and partially purified cAMP-dependent protein kinase with an activity of 0.2 nmol min^{-1} mL^{-1}. Under these conditions, maximal phosphorylation of glycogen synthase (1.3 mol of phosphate/10^5 g of glycogen synthase) was achieved after 30 min. Higher concentrations (3–10 nmol min^{-1} mL^{-1}) of the purified catalytic subunit of cAMP-dependent protein kinase produced a greater degree of phosphorylation (Figure 1A). After a rapid initial rate of phosphorylation ($\simeq$0.25 mol of phosphate/10^5 g of glycogen synthase per min) to 1.2 mol/10^5 g, there was a second, slow phase of phosphorylation ($\simeq$0.007 mol of phosphate/10^5 g of glycogen synthase per min) to a maximum of 1.84 mol/10^5 g of glycogen synthase after 90 min.

The additional slow phosphorylation was not due to contaminating substrates for protein kinase, introduced with the high concentrations of the catalytic subunit, since when glycogen synthase was omitted from the reaction mixture the amount of protein-bound phosphate was reduced by 93%. The

TABLE II: Kinetic Properties of Phosphorylated Glycogen Synthase.[a]

Phosphorylating enzyme	Expt	$A_{0.5}$ for Glc-6-P (μM)	n_H	−:+ Glc-6-P act. ratio	K_m for UDP-glucose (μM)
cAMP-dependent protein kinase	A	230	1.5	0.069	46
	B	250	1.5	0.050	
Synthase kinase	A	550	1.6	0.018	37
	B	380	1.7	0.030	
cAMP-dependent protein kinase + synthase kinase	A	1550	1.4	0.013	61
	B	2010	1.7	0.015	

[a] Glycogen synthase was phosphorylated for 90 min under the conditions described in the Experimental Procedures, except that 1 mM ATP was used under all conditions. In experiment A, glycogen synthase preparation $3a_1$ was used as substrate; in experiment B, glycogen synthase $3a_2$ was used as substrate. The K_m of glycogen synthase for UDP-glucose was determined in the presence of 7.5 mM Glc-6-P. Phosphate content was not determined in these experiments. Based on the data presented in Figures 1 and 3, these values would be 1.8 mol/10^5 g for the cAMP-dependent kinase, 1.1 mol/10^5 g for the synthase kinase, and 2.8 mol/10^5 g for the combination of kinases.

extent of phosphorylation of glycogen synthase by the catalytic subunit was not altered by increasing the ATP concentration from 0.2 to 1.0 mM. The heat-stable inhibitor of cAMP-dependent protein kinase completely blocked phosphorylation by the catalytic subunit.

Kinetic properties were determined for glycogen synthase phosphorylated for varying lengths of time with the catalytic subunit of cAMP-dependent protein kinase. Since the phosphorylated enzyme, like the I form, showed positive cooperativity, values for $A_{0.5}$ were determined from Hill plots. There was a gradual time-dependent increase in the $A_{0.5}$ of the enzyme for Glc-6-P which paralleled the increase in phosphate content (Figure 1B). The $A_{0.5}$ values were approximately 100 μM when the enzyme contained 1 mol of phosphate/10^5 g, and 250 to 300 μM when enzyme phosphorylation approached 2 mol of phosphate/10^5 g (Figures 1A and 2). The K_m of the phosphorylated glycogen synthase for UDP-glucose was also determined (Figure 1B). In contrast to the shift in affinity of the phosphorylated enzyme for Glc-6-P, the K_m for UDP-glucose (determined in the presence of 7.5 mM Glc-6-P) did not differ from that of the nonphosphorylated form of the enzyme even when 2 mol of phosphate was incorporated.

The activity ratio (−:+ Glc-6-P) decreased rapidly with incorporation of the first mole of phosphate and was 0.12 ± 0.002 after 5 or 10 min of phosphorylation. A further decrease to 0.06 ± 0.004 was seen at 30 min, and this ratio subsequently remained constant.

Phosphorylation with cAMP-Independent Synthase Kinase. Glycogen synthase I could also be phosphorylated with the cAMP-independent synthase kinase prepared from rabbit muscle. The activity of the synthase kinase preparation used in all phosphorylation experiments was that amount which catalyzed incorporation of 0.24 nmol of phosphate into casein min^{-1} mL^{-1}; the concentration of ATP was 1.0 mM. Maximal phosphorylation by this concentration of synthase kinase was 1.1 mol of phosphate/10^5 g of glycogen synthase (Figure 3). This value was reached between 30 and 60 min and maintained through 90 min of incubation. Higher concentrations of the synthase kinase preparation were not tested. The heat-stable inhibitor of protein kinase did not block phosphorylation at concentrations of 0.1, 0.3, and 1 mg/mL. When glycogen synthase was omitted from the phosphorylation mixture, the incorporation of phosphate into protein was decreased by 96%.

Analysis of the Glc-6-P activation kinetics of glycogen synthase phosphorylated with the cAMP-independent synthase kinase again revealed positive cooperativity (Figure 2). The $A_{0.5}$ values determined in two separate experiments (A and B) were 550 and 380 μM, respectively (Table II, Figure 2). The K_m for UDP-glucose in the presence of 7.5 mM Glc-6-P was unchanged by phosphorylation. Phosphorylation to 1 mol of phosphate/10^5 g by synthase kinase decreased the −:+ Glc-6-P activity ratio to a lower value than did phosphorylation to 2 mol/10^5 g by the catalytic subunit of cAMP-dependent protein kinase.

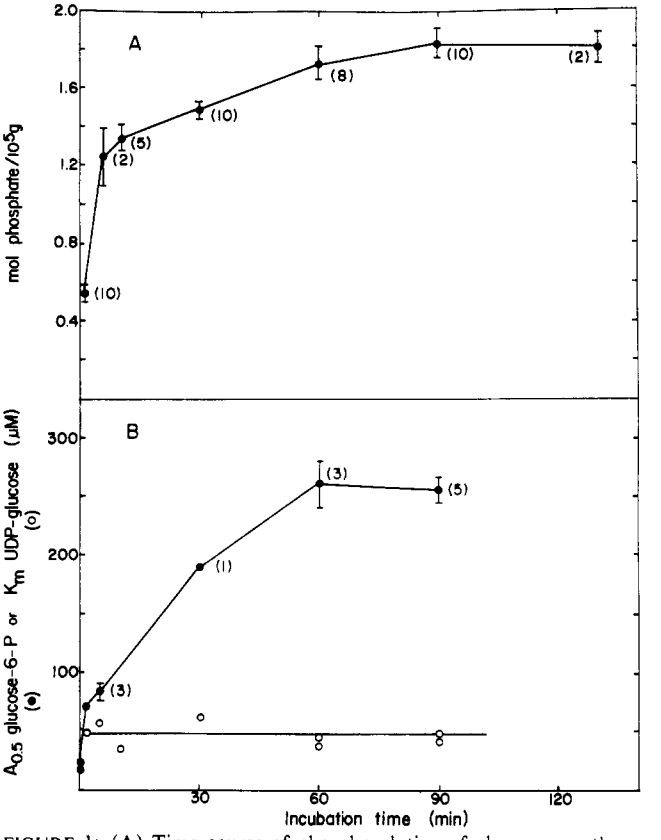

FIGURE 1: (A) Time course of phosphorylation of glycogen synthase preparations 2b and $3a_1$ with the catalytic subunit of cAMP-dependent protein kinase. Glycogen synthase was phosphorylated and phosphate incorporation into glycogen synthase determined as described under Experimental Procedures, using 0.2 mM ATP and 3–10 nmol min^{-1} mL^{-1} of catalytic subunit. The number of individual determinations and standard errors of the mean are indicated at each time point. (B) Changes in the $A_{0.5}$ for Glc-6-P (●) and in the K_m for UDP-glucose (in the presence of 7.5 mM Glc-6-P) (○) in phosphorylation experiments as described under A.

Phosphorylation of Glycogen Synthase with the Catalytic Subunit of cAMP-Dependent Protein Kinase Plus Synthase Kinase. When both catalytic subunit and cAMP-independent

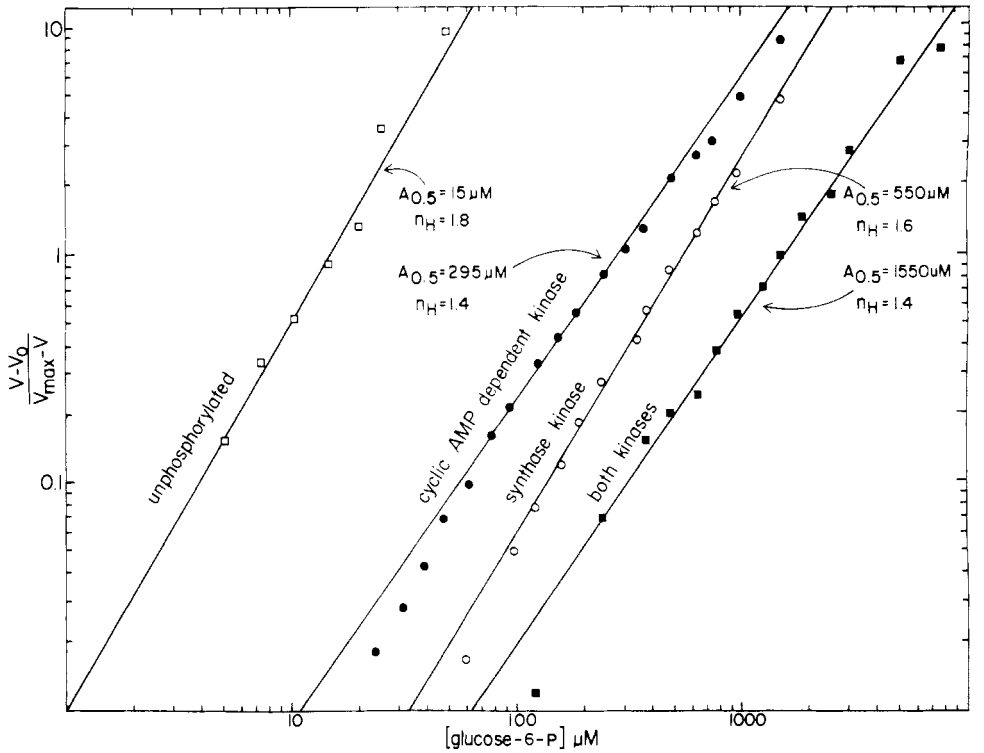

FIGURE 2: Representative Hill plots for glycogen synthase $3a_1$, before phosphorylation (□), after phosphorylation with the catalytic subunit of cAMP-dependent protein kinase for 90 min: (●; ~1.8 mol of phosphate/10^5 g), after phosphorylation with cAMP-independent synthase kinase for 90 min (○; ~1.1 mol of phosphate/10^5 g), and after phosphorylation with both the catalytic subunit and cAMP-independent synthase kinase (■; ~2.8 mol of phosphate/10^5 g) for 90 min. Phosphorylation conditions and glycogen synthase assay were as described under Experimental Procedures.

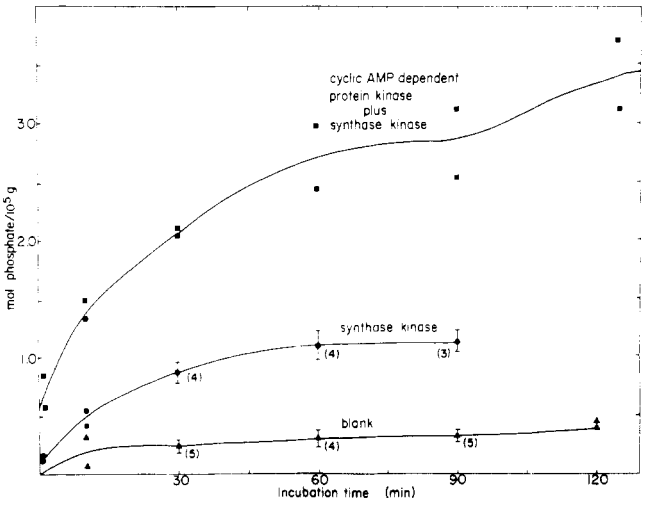

FIGURE 3: Glycogen synthase $3a_2$ was phosphorylated with 1 mM ATP, and 0.24 nmol min^{-1} mL^{-1} of synthase kinase alone (●) or in combination with the catalytic subunit of cAMP-dependent protein kinase, 3 nmol min^{-1} mL^{-1} (■) as described under Experimental Procedures. The blank (▲) represents Cl_3AcOH-insoluble phosphate incorporated into protein when both kinases were incubated in the absence of glycogen synthase.

TABLE III: Time Course of the Changes in Kinetic Properties of Glycogen Synthase Phosphorylated with cAMP-Dependent Protein Kinase Plus Synthase Kinase.[a]

Time (min)	Phosphate[c] (mol/10^5 g)	−:+ Glc-6-P act. ratio	$A_{0.5}$ for Glc-6-P (μM)
Control[b]	<0.10	0.640	17
1	0.85	0.050	
10	1.50	0.019	
30	2.04	0.017	900
60	2.43	0.016	1320
90	2.53	0.016	
120	3.11	0.012	1750

[a] Glycogen synthase $3a_2$ was phosphorylated as described under Experimental Procedures by incubation with a combination of the catalytic subunit of cAMP-dependent protein kinase and synthase kinase for the times indicated. [b] Glycogen synthase $3a_2$ in the I form, not incubated. The values are the same as those given in Table I. [c] Values were derived from an identical experiment done several days earlier using radioactive ATP. Determinations were made as described under Experimental Procedures.

synthase kinase were used to phosphorylate glycogen synthase, 3 mol of phosphate/10^5 g was incorporated in 90 min (Figure 3). Phosphorylation appeared to continue slowly up to 120 min. When glycogen synthase was omitted, protein in the mixture of kinases was phosphorylated to a greater extent than when either kinase was used alone (Figure 3).

The kinetic changes produced by phosphorylation of glycogen synthase with a combination of cAMP-dependent protein kinase and synthase kinase were compared to those produced by phosphorylation with each of the kinases used separately (Table II). Following a 90-min incubation with a combination of kinases, the $A_{0.5}$s of glycogen synthase for Glc-6-P were 1550 and 2010 μM, and the activity ratios were 0.013 and 0.015 in two separate experiments (Table II, Figure 2). The K_m for UDP-glucose was slightly but not significantly increased. In another experiment, glycogen synthase was phosphorylated with a combination of kinases and the $A_{0.5}$ and activity ratio were determined at various times (Table III). The $A_{0.5}$ for Glc-6-P increased progressively with phosphorylation while the −:+ Glc-6-P activity ratio decreased to 0.019 after 10 min of incubation and did not decrease significantly thereafter. The K_m for UDP-glucose determined in the presence of 7.5 mM Glc-6-P was 104 μM after a 120-min incubation.

Discussion

Several preparations of glycogen synthase purified from rabbit skeletal muscle in the I form have been shown to contain low amounts of alkali-labile phosphate, have high −:+ Glc-6-P activity ratios, and demonstrate positive cooperativity with respect to Glc-6-P. The molecular weight of the purified enzyme (268 000) suggested that the enzyme existed as a trimer of subunits with molecular weights of 88 000 to 90 000. Others have calculated molecular weights that are consistent with a tetrameric structure for the I form of glycogen synthase (Takeda et al., 1975; Soderling, 1976; Nimmo et al., 1976a). The different molecular weight we obtained may be the consequence of the duration and type of digestion of the glycogen pellet or the use of an affinity column as the final step of purification. Positive cooperativity of glycogen synthase with respect to Glc-6-P has also been noted by Roach et al. (1976), but was not seen by Nimmo et al. (1976b) who solubilized glycogen synthase from the glycogen particle with endogenous enzymes alone.

Glycogen synthase was phosphorylated by the addition of either cAMP-dependent or cAMP-independent protein kinase to the purified enzyme. Phosphorylation decreased the −:+ Glc-6-P activity ratio as well as the apparent affinity for Glc-6-P with no change in cooperativity with respect to Glc-6-P and with little change in the K_m for UDP-glucose determined in the presence of Glc-6-P. The cAMP-dependent protein kinase catalyzed the rapid incorporation of 1 mol of phosphate into the glycogen synthase subunit, accompanied by a marked decrease in the −:+ Glc-6-P activity ratio and a moderate increase in the $A_{0.5}$ for Glc-6-P. Slower incorporation of a second mole of phosphate increased the $A_{0.5}$ for Glc-6-P to a value 15 times that of the nonphosphorylated enzyme. Glycogen synthase was slowly phosphorylated to 1 mol of phosphate/10^5 g by the cAMP-independent synthase kinase at the concentration used. This was accompanied by a marked fall in the activity ratio and a marked increased in the $A_{0.5}$ for Glc-6-P. The combination of both kinases produced little further decrease in the −:+ Glc-6-P activity ratio but increased the $A_{0.5}$ for Glc-6-P to 90 times that of the nonphosphorylated glycogen synthase.

Several observations suggest that the sites on muscle glycogen synthase that were phosphorylated by the cAMP-independent synthase kinase were distinct from those phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Phosphate incorporation into glycogen synthase was additive when the kinase proteins were used in combination. The maximal phosphate incorporation produced by the catalytic subunit of cAMP-dependent protein kinase was 2 mol/10^5 g of glycogen synthase (Figure 1A), while that produced by the cAMP-independent kinase at the concentration used was 1 mol/10^5 g of glycogen synthase (Figure 3). When the kinases were used in combination, phosphorylation to 2 mol of phosphate/10^5 g was reached following a 30-min incubation, and phosphate incorporation continued beyond this time to values of 3 to 4 mol/10^5 g of glycogen synthase (Figure 3). In addition, phosphorylation with the cAMP-independent synthase kinase produced kinetic changes that were distinct from those seen following phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (Table II). The cAMP-independent synthase kinase alone catalyzed the conversion of glycogen synthase to a phosphorylated form that had greater dependence on Glc-6-P for activity (lower activity ratio) and a lower apparent affinity for Glc-6-P (higher $A_{0.5}$) than did glycogen synthase phosphorylated by the cAMP-dependent protein kinase alone. Additivity could be due to partial phosphorylation of the same sites by the two kinases. However, phosphorylation at identical sites is not consistent with the observation that incorporation of 2 mol/10^5 g by the cAMP-dependent kinase resulted in less change in activity ratio and $A_{0.5}$ than did phosphorylation to 1 mol/10^5 g with the synthase kinase.

Each of the 3 mol of phosphate incorporated into glycogen synthase produced distinct kinetic changes in the enzyme (Figure 1, Table III). Several reports have suggested that glycogen synthase is fully converted to the Glc-6-P-dependent form following phosphorylation to 2 mol of phosphate per subunit with cAMP-dependent protein kinase plus synthase kinase (Huang et al., 1975; Nimmo et al., 1976b). However, our data demonstrate that phosphorylation of glycogen synthase beyond 2 mol of phosphate/10^5 g produces additional kinetic changes that could be important in the regulation of glycogen synthase activity. Glycogen synthase phosphorylated to 2 mol/10^5 g of glycogen synthase following a 30-min incubation with both kinases had an $A_{0.5}$ for Glc-6-P of 900 μM and a −:+ Glc-6-P activity ratio of 0.017 (Table III). When phosphorylation was continued beyond 2 mol/10^5 g, the −:+ Glc-6-P activity ratio remained constant, but the $A_{0.5}$ for Glc-6-P increased further (1.5 to 2 mM). Itarte et al. (1977) also noted that the activity ratio did not decrease in parallel with phosphorylation of glycogen synthase. Thus, the −:+ Glc-6-P activity ratio is not an accurate or sensitive indicator of the activation state of multiply phosphorylated glycogen synthase.

Soderling (1975) has calculated that the intracellular concentration of cAMP-dependent protein kinase in skeletal muscle is sufficient to catalyze the incorporation of a second mole of phosphate into glycogen synthase. A number of reports in addition to our own demonstrate phosphorylation of skeletal muscle glycogen synthase by cAMP-independent synthase kinases (Huang et al., 1975; Nimmo et al., 1976b; Itarte et al., 1977), as well as by cAMP-dependent protein kinase or its catalytic subunit (Soderling, 1975; Huang et al., 1975; Itarte et al., 1977). High concentrations of cAMP-independent synthase kinase are found in a variety of tissues (Schlender and Reimann, 1977). Both cAMP-dependent protein kinase and cAMP-independent synthase kinase may be associated with the glycogen particle and copurify with glycogen synthase (Friedman and Larner, 1963; Villar-Palasi et al., 1971; Nimmo and Cohen, 1974; Nimmo et al., 1976b). Roach et al. (1976) examined the kinetic properties of preparations of rabbit skeletal muscle glycogen synthase which were phosphorylated by endogenous protein kinases in the course of purification. The most highly phosphorylated enzyme preparations contained from 3.2 to 3.5 mol of alkali-labile phosphate per subunit of glycogen synthase. The −:+ Glc-6-P activity ratios and apparent affinities for Glc-6-P of these preparations were similar to those seen in our experiments in which phosphorylation of muscle glycogen synthase to 3 mol/10^5 g was catalyzed by a combination of purified cAMP-dependent and cAMP-independent kinases. It is possible, therefore, that both cAMP-dependent and cAMP-independent synthase kinases are involved in the phosphorylation and regulation of glycogen synthase in vivo, with each enzyme producing unique effects, and the combination producing maximal inhibition of activity.

No conclusions about the regulation of glycogen synthase in vivo are warranted at present. Data on phosphorylation of purified glycogen synthase with purified kinases may be misleading with respect to conditions and relative enzyme concentrations that exist in the intact glycogen particle. Similarly, observations on molecular weight, the sites of phosphorylation,

and the associated alterations in the activation constant for Glc-6-P may be related to critical steps in the purification of glycogen synthase. Nevertheless, other studies from our laboratory indicate that several interconvertible Glc-6-P-dependent forms of glycogen synthase are present in intact adipose tissue (Kaslow and Mayer, manuscript in preparation) and that the phosphorylation state of glycogen synthase may be important in determining the rate at which glycogen synthase is converted to Glc-6-P independent activity by dephosphorylation (Brown et al., manuscript in preparation).

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