Phosphorylation of Rabbit Liver Glycogen Synthase by Multiple Protein Kinases*

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Purified rabbit liver glycogen synthase was found to be a substrate for six different protein kinases: (i) cyclic AMP-dependent protein kinase, (ii) two Ca²⁺-stimulated protein kinases, phosphorylation kinase (from muscle) and a calmodulin-dependent glycogen synthase kinase, and (iii) three members of a Ca²⁺ and cyclic nucleotide-independent class, PCO.7, FA/GSK-3, and casein kinase-1. Greatest inactivation accompanied phosphorylation by cyclic AMP-dependent protein kinase (to 0.5–0.7 phosphate/subunit, 5glucose-6-P activity ratio reduced from approximately 1 to 0.6) or FA/GSK-3 (to ~1 phosphate/subunit, activity ratio, 0.46). Phosphorylation by the combination Fₐ/GSK-3 plus PCO.7 was synergistic, and more extensive inactivation was achieved. The phosphorylation reactions just described caused significant reductions in the Vₘₐₓ of the glycogen synthase with little effect on the Sₜₐₜ (substrate concentration corresponding to Vₘₐₓ/2).

Phosphorylation kinase achieved a lesser inactivation, to an activity ratio of 0.75 at 0.6 phosphate/subunit. PCO.7 acting alone, casein kinase-1, and the calmodulin-dependent protein kinase did not cause inactivation of liver glycogen synthase with the conditions used. Analysis of CNBr fragments of phosphorylated glycogen synthase indicated that the phosphate was distributed primarily between two polypeptides, with apparent Mᵦ = 12,300 (CB-I) and 16,000–17,000 (CB-II). PCO.7 and casein kinase-1 displayed a decided specificity for CB-II, and the calmodulin-dependent protein kinase was specific for CB-I. The other protein kinases were able, to some extent, to introduce phosphate into both CB-I and CB-II. Studies using limited proteolysis indicated that CB-II was located at a terminal region of the subunit. CB-I contains a minimum of one phosphorylation site and CB-II at least three sites. Liver glycogen synthase is therefore potentially subject to the same type of multisite regulation as skeletal muscle glycogen synthase although the muscle and liver enzymes display significant differences in both structural and kinetic properties.

Multiple covalent phosphorylation is now well established as a feature of the hormonal control of rabbit skeletal muscle glycogen synthase (EC 2.4.1.11). Enzymological studies have identified a minimum of seven protein kinases able to phosphorylate the muscle enzyme at more than seven distinct sites (1–5). The protein kinases fall into three categories: (i) cyclic AMP-dependent protein kinase; (ii) Ca²⁺-stimulated enzymes of which two are currently known. One is phosphorylation kinase (6–11) and the second a calmodulin-dependent glycogen synthase kinase (12–14); and (iii) a Ca²⁺ and cyclic nucleotide-independent category that includes at least four members. Casein kinase-1 (15, 16) and PCO.7 (17–19), using our nomenclature, are casein and phosphotitin kinases that have been studied in various contexts besides glycogen synthase phosphorylation, and most likely correspond to the casein kinase I and casein kinase II categories as reviewed recently by Hathaway and Traugh (20). Another enzyme of this group, designated here Fₐ/GSK-3, was purified by Hemmings et al. (21) and appears identical with the phosphatase activating protein, Fₐ, of Merlevede and colleagues (22, 23). An additional member of this category, PCO.7 (24), has been less well characterized and was not included in the present study. Recent work using whole animals (25–30) or diaphragm muscles (31, 32) has provided evidence for a physiological role of the multiple phosphorylation of muscle glycogen synthase. Although many details remain to be worked out, multisite control by epinephrine and by insulin has been demonstrated (29–31).

Liver glycogen has a quite distinct physiological role as compared with muscle glycogen and, although some control inputs are common, its metabolism is governed by a different total set of regulatory stimuli. For example, a greater role for glucagon and perhaps glucose as well as the Ca²⁺-linked effects of α-adrenergic stimulation, vasopressin, and angiotensin II are controls potentially relevant to liver but not skeletal muscle glycogen metabolism (33, 34). One interesting question is to what extent such differences in regulation are expressed at the level of the interconversion of an intracellular target enzyme, like glycogen synthase, as compared with differences at the level of cell surface receptors and immediately receptor-linked events.

In order to address this type of question, an improved understanding of the enzymology and phosphorylation of liver glycogen synthase is necessary. Since the rabbit muscle enzyme is the best characterized, the important comparison, from the enzymological point of view, is with rabbit liver glycogen synthase. We have described the purification of this enzyme close to homogeneity (35, 36), as well as initial kinetic characterization of the enzyme purified either as a dephosphorylated, glucose-6-P-independent form or else purified to maintain its endogenous covalent phosphate content (approximately 5 phosphates/subunit) (36). The phosphorylated enzyme had a much lower Vₘₐₓ when the substrate UDP-glucose

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was varied but a similar $S_0$ for UDP-glucose. The phosphorylated enzyme was also much more susceptible to activation by glucose-6-P, as reflected in a $\pm$glucose-6-P activity ratio of 0.02-0.05 (36). In the present study, enzyme purified in a relatively dephosphorylated state (<0.6 phosphate/subunit) was subjected to phosphorylation by six different protein kinases. Most effective in inactivating liver F$_x$/GSK-3 and cyclic AMP-dependent protein kinase. The primary phosphorylation sites of liver glycogen synthase were distributed between two CNBr fragments, one of which corresponds to a terminal region of the subunit. Although possessing several important similarities to the skeletal muscle enzyme, rabbit liver glycogen synthase also displays significant structural differences.

**EXPERIMENTAL PROCEDURES**

**Glycogen Synthase**—The purification of liver glycogen synthase followed the methods described previously (36) for the dephosphorylated form (I form) of the enzyme, with one modification. Glycogen, 10% (w/v), was included in the buffer used to elute the enzyme from the DEAE-cellulose column. The inclusion of glycogen significantly improved the stability of the enzyme at this stage. The resulting glycogen synthase properties compared well to those described by Cohen (38), with an alkali-labile phosphate content less than 0.6 phosphate/subunit and an activity ratio (see later) of 0.9-1.1. The enzyme contained approximately 30 mg/ml of endogenous glycogen. Rabbit skeletal muscle glycogen synthase was purified by published procedures (37).

**Protein Kinases**—Phosphorylase kinase was purified from rabbit muscle by a slight modification of the method of Cohen (38). The catalytic subunit of cardiac cyclic AMP-dependent protein kinase was kindly supplied by Dr. David Braunig, Brown University. PC$_{37}$ (18), F$_y$/GSK-3 (16), the calmodulin-dependent glycogen synthase kinase (13), and casein kinase-1 (16) were purified from rabbit liver by procedures described previously and were contained in the buffers described in the corresponding references. Cyclic AMP-dependent protein kinase was diluted into 50 mM Tris-HCl, pH 7.5; 25 mM P$_2$-mercaptoethanol, 10.5 mg/ml rabbit liver glycogen, 0.0375 to 75 mM Na$_2$SO$_4$, 15 mM KF, 25 mM P-glycerophosphate, pH 8.2.

**Assay of Glycogen Synthase**—Glycogen synthase activity was measured by the incorporation of $^3$H from UDP-$[^3$H]glucose into glycogen. The standard assay followed the method of Thomas et al. (36) and involved a dilution of the enzyme sample 1:3 in the final reaction mixture. The reaction mixture (100 pl) contained 25 mM Tris-HCl, pH 7.5; (i) 66 pl of liver or muscle glycogen synthase at a concentration of 1 unit/ml F$_x$/GSK-3, 12 pl of 2 mM/ml calmodulin-dependent protein kinase, 12 pl of 85 mg/ml phosphatase, 21 pl of 5 mM myosin casein kinase-1, or 12 pl of 90 mg/ml cyclic AMP-dependent protein kinase. Total reaction volume was 100 pl. The activity ratio is the ratio of glycogen synthase activity in the presence of 7.2 mM glucose-6-P to that measured in the presence of 0.65 mM EGTA, 50 mM 6-mercaptoethanol, 10% (v/v) glycerol, pH 7.6. No loss of activity was observed after dialysis. The rabbit muscle enzyme was diluted into the above buffer plus 30 mg/ml of rabbit liver glycogen, 0.65 mg/ml EDTA, 0.65 mM EGTA, 50 mM P$_2$-mercaptoethanol, 10% (v/v) glycerol, pH 7.6.

**Phosphorylation**—The quantitation of glycogen synthase phosphorylation was as described previously (36). Typically, the final reaction volume was 100 pl and was composed of (i) $\pm$ a solution containing 1.2 mM $[^3$P]ATP (specific activity, 20-30 cpm/pmol); 0.35 mM Mg$_2$ATP, 0.35 mM Mg$_2$EGTA, pH 7.5; (ii) 66 pl of liver or muscle glycogen synthase at a concentration of 0.13 mg/ml; (iii) where indicated, 12 pl of 7 mg/ml PC$_{37}$, 12 pl of 1 unit/ml F$_x$/GSK-3, 12 pl of 2 mg/ml calmodulin-dependent protein kinase, 12 pl of 85 mg/ml phosphatase, 21 pl of 5 mM myosin casein kinase-1, or 12 pl of 90 mg/ml cyclic AMP-dependent protein kinase. The final concentration of ATP was 100 pl to each of the above solutions. The final reaction volume was altered, the above proportions were respected). When phosphorylation kinase was present, a final concentration of 0.9 mC CaCl$_2$ was included. When the calmodulin-dependent protein kinase was present, 0.9 mg/ml CaCl$_2$ and 12 pg/ml of calmodulin were included. In tests of additivity of phosphorylation by PC$_{37}$ and F$_x$/GSK-3, where a protein kinase was omitted, the appropriate buffer was substituted. Liver glycogen synthase was contained in the buffer of the final stage of its purification, namely, 50 mM $\beta$-glycerophosphate, 5 mM EDTA, 2 mM dithiothreitol, 0.2 M glucose, pH 7.6. Muscle glycogen synthase was sufficiently concentrated that it could be diluted (approximately 50-fold) into the above buffer without significant alteration in the buffer composition. Rabbit liver glycogen, to a concentration of 30 mg/ml, was added to the diluted muscle enzyme (free of glycogen as purified) to approximate the endogenous glycogen present in the liver glycogen synthase. The objective was to allow as valid a comparison as possible between the phosphorylation of liver and muscle glycogen synthases by different protein kinases.

**Two protein kinases posed additional problems.** With the conditions just described, casein kinase-1 and the calmodulin-dependent protein kinase were relatively ineffective in phosphorylating even muscle glycogen synthase. To contrive reaction conditions closer to those used in previous studies of the calmodulin-dependent protein kinase (13), liver glycogen synthase was diluted into a buffer containing three changes, against 1 liter of 50 mM Tris-HCl, 1.75 mM EDTA, 0.65 mM EGTA, 50 mM $\beta$-mercaptoethanol, 10% (v/v) glycerol, pH 7.6. No loss of activity was observed after dialysis. The rabbit muscle enzyme was diluted into the above buffer plus 30 mg/ml of rabbit liver glycogen. For casein kinase-1, we traced the problem to the presence of glycogen and $\beta$-glycerophosphate, both of which inhibited protein kinase activity. We could not eliminate the presence of glycogen in the liver enzyme, although $\beta$-glycerophosphate was removed by the dialysis just described. Thus, dialyzed liver enzyme and muscle enzyme diluted in the above buffer were used for studies of casein kinase-1 action (see also under "Results").

**Proteolysis and CNBr Fragmentation of Liver Glycogen Synthase**—Liver glycogen synthase (2.7 to 4.4 mg), phosphorylated by a given protein kinase using $[^3$P]ATP, was precipitated with 10% (w/v) trichloroacetic acid with the addition of 5 mg of bovine serum albumin. After standing for 30 min, the precipitate was recovered by centrifugation and dissolved in 70% (v/v) formic acid. Excess CNBr was added, normally for 3 h, and the solution was evaporated to dryness with a centrifugal evaporator (SpeedVac, Savant). From trial variations in the time of exposure to CNBr, it was established that, at least for $^{32}$P-labeled fragments, cleavage was complete at 3 h.

In the experiments, liver glycogen synthase treated with CNBr was exposed to proteases before CNBr cleavage. The phosphorylation reaction was terminated by the addition of EDTA to a final concentration of 20 mM. Trypsin (5 mg/ml) or chymotrypsin (5 mg/ml) was added, as indicated, to the glycogen synthase, present at a concentration of 44 mg/ml. In control incubations, water substituted for the protease. After 10 min at 30 °C, diluted solutions containing 1.5 mg of glycogen synthase were removed. One was precipitated with trichloroacetic acid to be subjected to CNBr cleavage as described above.
The second sample was made 70% (v/v) with respect to formic acid and evaporated to dryness with a centrifugal evaporator. Samples were analyzed by gel electrophoresis.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using 0.75-mm-thick slab gels, followed a slight modification of the method of Laemmli (41) as described previously (42). The native or proteolytically degraded subunit was analyzed on 8% acrylamide gels. These gels were stained with the silver technique (43) and dried between cellulose sheets. CNBr fragments were analyzed on gradient gels formed of equal volumes of 6% and 26% acrylamide solutions. These gels were dried, without staining, onto filter paper. Autoradiograms were made by placing dried gels in contact with Cronex 4 x-ray film (DuPont).

Apparent molecular weights for polypeptides (defined as $M_{app}$) were estimated from the migration of standard proteins: phosphorylase b (94,000), bovine serum albumin (45,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (21,000), myoglobin (17,000), and a CNBr fragment of myoglobin (14,400). Plots of the logarithm of molecular weight versus relative mobility were essentially linear in this range. Linear extrapolation of the standard curve was used to estimate some values of $M_{app}$ below 14,400. Even though the calibration is probably less correct in this region, objective evaluation of electrophoretic behavior is achieved. The term $M_{app}$ is used because in some instances the phosphorylation of polypeptides in this study altered electrophoretic mobility (see also Ref. 42).

**Other Materials and Methods**—Calmodulin was purified to homogeneity from rabbit skeletal muscle by a variation of the method of Doddrell et al. (44). Trypsinogen, treated with L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone, was from Millipore. Chymotrypsin (treated with N-acetyl-L-lysine chloromethyl ketone) was from Sigma. Rabbit liver glycogen (Sigma Type III) was deionized before use as described previously (45). Chemicals for electrophoresis were from Bio-Rad. CNBr was from Aldrich. [γ-32P]ATP was from Amer sham-Searle. Other chemicals were of reagent grade.

Protein concentration was determined by the Coomassie blue binding assay (46), using bovine serum albumin as standard. The concentration of [γ-32P]ATP was determined spectrophotometrically.

**RESULTS**

**Phosphorylation of Rabbit Liver Glycogen Synthase**—The phosphorylation of liver glycogen synthase by six different protein kinases was investigated. These six enzymes had been defined as glycogen synthase kinases on the basis of their phosphorylation of rabbit muscle glycogen synthase. Therefore, parallel reactions were run to monitor the phosphorylation of both liver and muscle glycogen synthase under conditions that were as far as possible identical (see "Experimental Procedures"). The phosphorylation of muscle glycogen synthase thus served as a positive control. Initially, the effects of phosphorylation on enzyme activity were screened by measurement of the $\Delta$glucose-6-P activity ratio, which was shown, in previous work (35, 36), to be a useful index of the interconversion of rabbit liver glycogen synthase. Even in quite prolonged incubations (up to 120 min), the rabbit liver glycogen synthase activity was stable, and also little or no alteration in the activity ratio was observed. In the presence of glycogen, the muscle enzyme was also stable in these incubations.

PC$_{0.7}$ phosphorylated both liver and muscle glycogen synthase to 0.3-0.4 phosphate/subunit, without decreasing the activity ratio of either enzyme (Fig. 1). F$_A$/GSK-3, acting alone, phosphorylated liver glycogen synthase maximally to a level of approximately 1.2 phosphates/subunit, 0.7 phosphate/subunit in the incubation shown in Fig. 1. Such levels are somewhat lower than for the phosphorylation of the muscle enzyme. The action of F$_A$/GSK-3 caused a significant reduction in the activity ratios of both liver and muscle glycogen synthase (Fig. 1). When liver glycogen synthase was exposed to the combination of PC$_{0.7}$ plus F$_A$/GSK-3, a synergistic phosphorylation was observed (Fig. 1), more phosphate being introduced than predicted from the individual actions of the protein kinases. A correspondingly greater inactivation was also found. A similar synergistic phosphorylation of muscle glycogen synthase by these two enzymes had been previously reported (42), as is shown also in Fig. 1. As described in an earlier report (36), phosphorylation by F$_A$/GSK-3 caused a reduction in the electrophoretic mobility of the liver glycogen synthase subunit. The subunit of $M_{app} = 85,000$ was converted, via a species of $M_{app} = 88,000$, to one of $M_{app} = 90,000$. An almost identical scheme has been described for muscle glycogen synthase (42).

Phosphorylation of liver glycogen synthase by cyclic AMP-dependent protein kinase proceeded to 0.5–0.7 phosphate/subunit, a stoichiometry significantly lower than that achieved for the muscle enzyme (Fig. 2). This phosphorylation of liver glycogen synthase correlated with a reduction in the activity ratio to 0.6. We also noted that the phosphorylation of rabbit liver glycogen synthase by cyclic AMP-dependent protein kinase caused a reduction in electrophoretic mobility to an $M_{app} \approx 90,000$, as described above for F$_A$/GSK-3 action (not shown).

Phosphorylation kinase, from muscle, phosphorylated both liver and muscle glycogen synthase to similar extents, 0.6 phosphate/subunit (Fig. 2). Whereas significant inactivation of the muscle enzyme accompanied this phosphorylation, only modest inactivation of liver glycogen synthase, to an activity ratio of 0.75, occurred.

The calmodulin-dependent glycogen synthase phosphorylated liver glycogen synthase to a stoichiometry of approximately 0.6 phosphate/subunit (Fig. 2). In a parallel reaction, the muscle enzyme incorporated 1.2 phosphates/subunit (Fig. 2) although levels approaching 2 phosphates/subunit have been obtained in other studies (13). The phosphorylation of the muscle enzyme corresponded to a reduction in the activity ratio to 0.5, consistent with earlier experiments (13). However, the introduction of 0.6 phosphate/subunit into liver glycogen synthase had no effect on the activity ratio under these conditions.

**FIG. 1.** Phosphorylation of glycogen synthase by F$_A$/GSK-3 and/or PC$_{0.7}$. Liver (B) or muscle (A) glycogen synthase were phosphorylated by F$_A$/GSK-3, PC$_{0.7}$, or both protein kinases together, as described under "Experimental Procedures." At the indicated times, samples were removed to measure phosphate incorporation (filled symbols) or were diluted for measurement of the activity ratio by the standard assay (open symbols). Incubations were: with PC$_{0.7}$ alone (C, D), F$_A$/GSK-3 alone (E, F), F$_A$/GSK-3 plus PC$_{0.7}$ (G, H), no kinase (I). The level of phosphorylation, predicted for the combination PC$_{0.7}$ plus F$_A$/GSK-3 on the basis of simple additivity, is shown by ---.
Evaluation of the action of casein kinase-1 on liver glycogen synthase was complicated by the inhibitory effect of glycogen on the activity of this protein kinase (16). Thus, in the parallel phosphorylation of muscle glycogen synthase, a much slower reaction was observed (Fig. 2) than can be obtained in the absence of glycogen. Nonetheless, casein kinase-1 was able to introduce up to approximately 1 phosphate/subunit into liver glycogen synthase, although the reaction did not appear to be complete (Fig. 2). Little if any alteration in the activity ratio of liver glycogen synthase was observed. In fairness, though, with the muscle enzyme, casein kinase-1 does not cause very great inactivation at a phosphorylation level of 1 phosphate/subunit, even though significant inactivation can be observed at higher phosphorylation stoichiometries (an activity ratio of 0.35 at 4 phosphates/subunit; Ref. 16). Phosphorylation by casein kinase-1 was a third instance in which a reduction in the electrophoretic mobility of the liver glycogen synthase subunit, to \( M_{\text{sub}} \approx 90,000 \), occurred (not shown).

The effects of the different protein kinases on the inactivation of rabbit liver glycogen synthase are summarized in Fig. 3 where activity ratio is plotted as a function of the phosphorylation introduced by the different protein kinases. Most effective inactivation, as noted above, was associated with phosphorylation by cyclic AMP-dependent protein kinase or by \( F_{\alpha}/GSK-3 \) (with or without \( P_{\text{C0.7}} \)). In all these cases, the activity phosphorylation plots were nonlinear, a phenomenon that may be related to the modification of multiple phosphorylation sites (see below).

**Effects of Phosphorylation on Kinetic Properties of Liver Glycogen Synthase**—In the initial screening, only the activity ratio was monitored as a function of phosphorylation. For the most effective phosphorylations, a more detailed analysis of the effects on the kinetic behavior of the enzyme was made. With liver glycogen synthase phosphorylated to 0.7 phosphate/subunit by cyclic AMP-dependent protein kinase, the dependence of the activity on substrate, UDP-glucose, or activator, glucose-6-P, concentrations was determined (Fig. 4). This phosphorylation caused an almost 3-fold reduction in the \( V_{\text{max}} \) obtained from variation of UDP-glucose concentration without major alteration in the \( S_{0.5} \) for UDP-glucose (Table I). The Hill slope was not greatly affected, although, from Fig. 4, it is apparent that the phosphorylated enzyme may have become susceptible to substrate inhibition at elevated UDP-glucose levels. The main effect of this phosphorylation on the glucose-6-P response was to increase the extent to which the sugar phosphate could stimulate activity. At high glucose-6-P levels, the difference in activity between the control and the phosphorylated enzyme was not so marked. A comparable effect on the +glucose-6-P activity in the standard assay was also observed in the experiments of Fig. 2 but was not included so as to simplify the figure.

Analogous experiments were performed with liver glycogen synthase first phosphorylated with \( F_{\alpha}/GSK-3 \) or \( P_{\text{C0.7}} \) plus \( F_{\alpha}/GSK-3 \) (Fig. 4). Phosphorylation by \( F_{\alpha}/GSK-3 \) caused qualitatively similar changes in kinetic properties as those just described for cyclic AMP-dependent protein kinase action. However, quantitatively, phosphorylation by \( F_{\alpha}/GSK-3 \) was more potent, causing a greater decrease in the \( V_{\text{max}} \) and a greater increase in the extent to which glucose-6-P could stimulate activity (Table I). The synergistic phosphorylation by \( P_{\text{C0.7}} \) and \( F_{\alpha}/GSK-3 \) correlated with even greater effects on these parameters (Fig. 4, Table I). The phosphorylation did not significantly alter the \( S_{0.5} \) for UDP-glucose.

**Analysis of the Site Specificity of Liver Glycogen Synthase Phosphorylation by the Use of CNBr Cleavage**—Liver glycogen synthase, phosphorylated as in the various reactions described above, was also subjected to cleavage with CNBr and the
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Fig. 4. Effects of phosphorylation of the enzyme kinetic behavior of liver glycogen synthase. Glycogen synthase was phosphorylated to 0.7 phosphate/subunit by cyclic AMP-dependent protein kinase (C, A and B), to 1.2 phosphates/subunit by Fα/GSK-3 (Δ, C and D), or to 1.7 phosphates/subunit by PCα7 plus Fα/GSK-3 (Δ, C and D). Nonphosphorylated enzyme was analyzed as a control (●). The activity of the glycogen synthase as a function of UDP-glucose concentration (A and C) or glucose 6-P concentration (B and D) was determined as described under "Experimental Procedures."

resulting fragments were separated on 6-20% gradient polyacrylamide gels in the presence of sodium dodecyl sulfate (Figs. 5 and 6). Autoradiograms were made to identify phosphorylated polypeptides. PCα7 caused the introduction of two 32P-labeled fragments of 17,000 were visible (Fig. 5, track 9), but with increasing phosphorylation, the higher mobility fragment was no longer detected and only the species of Mapp = 17,000 remained. This result suggests that the two species, Mapp = 16,000 and 17,000, represented a single polypeptide whose electrophoretic mobility was reduced if phosphorylated by Fα/GSK-3. This CNBr fragment is designated CB-II. This analysis also indicated that the synergistic phosphorylation by PCα7 and Fα/GSK-3 acting together occurred in CB-II (see Fig. 5). With the combined action of PCα7 and Fα/GSK-3, the other phosphorylated CNBr fragment, Mapp = 12,300, was present with unchanged mobility; this fragment is termed CB-I. We should note also the presence of a small amount of 32P running with Mapp = 19,000 (Fig. 5, track 12). In Fig. 5 is also shown the electrophoretic separation of the two 32P-labeled CNBr-fragments derived from rabbit muscle glycogen synthase.3

For glycogen synthase phosphorylated by the calmodulin-dependent protein kinase, nearly all of the phosphate was associated with a species electrophoretically identical with CB-I (Fig. 6). Phosphorylase kinase caused labeling of species of Mapp = 16,000 and 12,300, the phosphorylation of the latter occurring somewhat more rapidly (Fig. 6). In this case, a minor phosphorylated fragment of Mapp = 14,000 was also detected. Cyclic AMP-dependent protein kinase caused initial phosphorylation in the species of Mapp = 12,300 with a more gradual introduction of phosphate into a polypeptide of Mapp = 17,000. From examination of the activity-phosphorylation curve for this phosphorylation reaction (Fig. 3), it would appear that it is the introduction of phosphate into the larger CNBr fragment that correlates best with inactivation. Casein kinase-1 action was relatively specific for the introduction of phosphate into a CNBr fragment of Mapp = 17,000. Our hypothesis is that the phosphorylation sites for the above protein kinases are contained primarily in two CNBr fragments, CB-I (Mapp = 12,300) and CB-II (Mapp = 16,000-17,000), of rabbit liver glycogen synthase.

It should be noted that the electrophoretic mobility of the larger phosphorylated fragment of muscle glycogen synthase, CB-2, is also very dependent on its exact phosphorylation state (42). Shown in Fig. 5 is the polypeptide running with its maximum mobility, with Mapp = 22,400.

TABLE I

| Property | I form*+ | Enzyme* phosphorylated by | D form* |
|----------|---------|----------------------------|--------|
|            |         | cAMP PK                   | Fα/GSK-3 | PCα7 + Fα/GSK-3 |
| Standard assay | +Glucose-6-P (units/mg) | 15-17 | 12.2 | 9.6 | 6.6 | 3-5 |
| Phosphorylation (phosphates/subunit) | <0.6 | +0.7d | +1.2d | +1.7d | 5.2 |
| UDP-glucose varied | Vmax (units/mg) | 21-23 | 8.0 | 6.3 | 4.0 | 0.39 |
| Hill slope | 1.2 | 1.3 | 1.9 | 3.0 | 2.1 |
| Glucose-6-P varied | Vmax (units/mg) | 10.5-11.5 | 8.6 | 6.2 | 0.9 | 0.8 |
| Mapp (mM) | 0.1-0.2 | 0.30 | 0.38 | 0.48 | 7.2 |
| Hill slope | 0.71 | 0.71 | 0.80 | 1.03-1.5 |

* Data from Refs. 35 and 36.
+ Data from present report.
+ cAMP PK, cyclic AMP-dependent protein kinase.
+ Phosphate introduced by the indicated protein kinase(s).
+ Estimate approximately (see Fig. 4).
Calmodulin-dependent protein kinase or limited action of cyclic AMP-dependent protein kinase were used to label preferentially CB-I (Fig. 7). Neither trypsin nor chymotrypsin action had effects on the $^{32}$P-associated with CB-I commensurate with the total degradation of the starting subunit. Parallel experiments utilized glycogen synthase phosphorylated by casein kinase-I so as to label CB-II. In this case, complete loss of the $^{32}$P-labeled CB-II was seen after proteolysis with either trypsin or chymotrypsin (Fig. 7). The same loss of the trace of CB-II from enzyme phosphorylated by cyclic AMP-dependent protein kinase can be seen in tracks 2 and 3 of Fig. 7. Similar results were also obtained if the CB-II had been labeled by the action of PCo.7 (not shown). Therefore, CB-II is located terminally and CB-I is still contained in the degraded subunit generated either by trypsin or chymotrypsin.

**DISCUSSION**

From the present investigation, we can conclude that rabbit liver glycogen synthase is a substrate in vitro for the six protein kinases tested. The dominant phosphorylation sites are distributed between two CNBr fragments, at least one of which is located terminally in the glycogen synthase subunit, as is depicted in Fig. 8. We are uncertain as to the significance of the two minor phosphorylated CNBr fragments of $M_{app} = 14,000$ and 19,000. Their presence was not altered by increasing the time of exposure to CNBr so that it is unlikely that they result from incomplete cleavage. Three of the protein kinases, the calmodulin-dependent protein kinase, PCo.7, and casein kinase-I, were relatively specific for one or another of CB-I and CB-II. The three other protein kinases phosphorylated both CB-I and CB-II. The smaller fragment CB-I was subject to phosphorylation by four of the protein kinases although the present data do not indicate whether or not CB-I contains more than one phosphorylation site. The results do indicate, however, that CB-II contains multiple phosphorylation sites. First, the detection of CB-II with $M_{app}$ of either
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![Diagram](image)

**FIG. 8.** Map of the phosphorylation of the rabbit liver glycogen synthase subunit. The presence of two primary phosphorylated CNBr fragments of the subunit is indicated by the boxes. The numbers represent the apparent molecular weights of the fragments (×10⁶) following phosphorylation by the indicated protein kinase. Thus, CB-I has $M_{app} = 12,300$ and CB-II $M_{app} = 16,000$ or 17,000 depending on its phosphorylation. CB-II is located toward one end of the molecule. The arrow linked to C,T indicates that CB-II contains sites of the native enzyme, that are cleaved by trypsin and chymotrypsin. Abbreviations for protein kinases are as in Fig. 2.

16,000 or 17,000 is evidence of at least two distinct phosphorylation sites. Second, phosphorylation of CB-II such as to alter its electrophoretic mobility to an $M_{app} = 17,000$ occurred either with (action of cyclic AMP-dependent protein kinase or F₆/GSK-3) or without (action of casein kinase-1) inactivation of the glycogen synthase. Thus, as a conservative estimate, one can define a minimum of three phosphorylation sites in CB-II. The number could be greater if F₆/GSK-3 and cyclic AMP-dependent protein kinases were specific for different sites in CB-II. The results of limited proteolytic cleavage of the subunit, either with trypsin or chymotrypsin, indicated that CB-II must be located at one end of the polypeptide chain. The same approach did not reveal whether or not CB-I was located terminally, and showed only that electrophoretically unaltered CB-I could be recovered from the subunit that had been cleaved to $M_{app} = 72,000$–80,000. Further work is required to define more precisely both the number and distribution of the phosphorylation sites.

Another aspect of the phosphorylation of liver glycogen synthase is deserving of mention. The combined action of PC₅₀ and F₆/GSK-3 resulted in a synergistic introduction of two or three phosphate groups.

An important part of this investigation was to evaluate the effectiveness of the various protein kinases in inactivating liver glycogen synthase, and perhaps to identify those most likely to have physiological roles. The initial screening followed simply the activity ratio, which we have shown previously to be a good index of the difference in kinetic properties between the "dephosphorylated" form of the enzyme (I form) and enzyme purified to maintain its endogenous phosphate, of approximately 5 phosphates/subunit (36). Of the protein kinases tested, it was evident that F₆/GSK-3 and cyclic AMP-dependent protein kinase were by far the most potent in inactivating glycogen synthase. Phosphorylation by both of the Ca²⁺-dependent enzymes caused no or very little reduction in the activity ratio. Of course, in this kind of experiment, negative results are not unequivocal, even though we tried to arrange positive controls in the form of parallel inactivations of the muscle enzyme. Nonetheless, we are forced to conclude that, at least with the conditions used, neither phosphorylase kinase nor the calmodulin-dependent protein kinase could be considered a potent inactivator of liver glycogen synthase. The results were, in a sense, unexpected since these two Ca²⁺-activated enzymes are potential candidates to mediate Ca²⁺-dependent inactivation of glycogen synthase in isolated hepatocytes through the actions of α-adrenergic agonists, angiotensin II, or vasopressin (see Refs. 33 and 34 for a review). We cannot, of course, exclude totally the possibility that appropriate conditions to reveal inactivation were not used or that some significant property, other than catalytic activity, was modified.

Negative results were also obtained with casein kinase-1 in that phosphorylation close to 1 phosphate/subunit was without effect on activity. Evaluation of this protein kinase is more difficult since its inactivation of muscle glycogen synthase is only significant at rather high levels of phosphorylation, perhaps 3–4 phosphates/subunit. Such levels of phosphorylation were never achieved using liver glycogen synthase as a substrate, possibly because of the presence of glycogen, an inhibitor of casein kinase-1 (16).

Most extensive inactivation of liver glycogen synthase was achieved through phosphorylation with F₆/GSK-3. PC₅₀ action, which on its own did not inactivate the enzyme, did result in a synergistic phosphorylation in combination with F₆/GSK-3 and a greater degree of inactivation. Since this synergistic phosphorylation was directed toward CB-II, a role for CB-II site(s) in causing the inactivation is suggested. Cyclic AMP-dependent protein kinase caused phosphorylation first in CB-I but again it appeared that it was the subsequent phosphorylation in the region of CB-II that correlated with inactivation of the glycogen synthase. From more detailed kinetic analyses of glycogen synthase phosphorylated by these enzymes, it was evident that the primary effects were on $V_{max}$ and on the extent of activation by glucose-6-P. The $S_{0.5}$ for UDP-glucose was relatively unchanged. Such effects of phosphorylation on enzyme kinetic parameters were qualitatively similar to those inferred from the study of enzyme purified to maintain its endogenous phosphorylation state of approximately 5 phosphates/subunit (36, 36). However, the degree of inactivation of enzyme phosphorylated in vivo in the present study was not as great. This fact is particularly apparent from examination of the values of $V_{max}$ for varied UDP-glucose or the activity ratio in Table I. Also, in vitro phosphorylation was relatively ineffective in increasing the $M_{app}$ for glucose-6-P. It is likely that phosphorylation in vivo involves the combined action of multiple protein kinases and higher levels of phosphorylation are needed to achieve more complete inactivation. Nonetheless, we can conclude that the effects of phosphorylation by cyclic AMP-dependent protein kinase and F₆/GSK-3 partially mimic the inactivation occurring in vivo.

Insofar as one may judge from the enzymology, therefore, cyclic AMP-dependent protein kinase and F₆/GSK-3 (perhaps in conjunction with PC₅₀) are the primary candidates to control liver glycogen synthase activity. A role for cyclic AMP-dependent protein kinase is indeed consistent with cyclic AMP-mediated regulation of glycogen synthase by glu-
cagon or β-adrenergic agonists (33, 34). The mechanisms of regulating P_{i}/GSK-3 and P_{G}, in vivo are not known but could be important for the control of liver glycogen synthase. A role for P_{i}/GSK-3 in mediating the effects of insulin (30) and epinephrine (29) on muscle glycogen synthase has been suggested. Certainly, it is reasonable to propose that the regulation of liver glycogen synthase also involves multiple phosphorylation reactions.

Another consequence of the present study is that we can extend the comparison between liver and muscle glycogen synthases started in previous work (35, 36). The enzymes share several properties: (i) subunit of $M_{s}$ of $\sim 85,000$, whose electrophoretic mobility can be reduced, upon appropriate phosphorylation, to give an $M_{s}$ of $\sim 90,000$; (ii) activation by glucose-6-P, to a greater extent when appropriately phosphorylated; (iii) phosphorylation by the six different protein kinases studied in the present report at multiple sites, distributed mainly between two CNBr fragments; (iv) in general terms, a correlation between phosphorylation and inactivation; and (v) synergistic phosphorylation by the enzymes P_{G} and P_{i}/GSK-3.

One of the principal differences between the muscle and liver enzymes concerns the way in which phosphorylation is translated into reduced activity. Whereas inactivation of the muscle enzyme is predominantly a result of increasing the $S_{0.5}$ for UDP-glucose, inactivation of liver glycogen synthase can be explained as a reduction in $V_{m}$. An even more fundamental difference between the two enzymes is revealed by examination of CNBr fragments of the phosphorylated enzymes. The phosphorylated CNBr fragments from the two enzymes indicated quite distinctive patterns when analyzed by polyacrylamide gel electrophoresis. This result indicates differences at the level of primary structure and rabbit liver and muscle glycogen synthases must be viewed as isozymes, the products of separate genes. One may hypothesize, therefore, that, although many broad features of the regulation by phosphorylation are conserved in the two tissues, the differences in kinetic properties might reflect evolved specializations relevant to the individual tissues. Although precise rationalization of the results is difficult at this time, some interesting questions concerning the comparative control mechanisms of glycogen synthesis in liver and muscle are raised.

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REFERENCES
1. Roach, P. J. (1981) Curr. Top. Cell. Regul. 20, 45–105
2. Cohen, P. (1982) Nature (Lond.) 296, 613–620
3. Soderling, T. R. (1979) Mol. Cell. Endocrinol. 16, 157–179
4. Preiss, J., and Walsh, D. A. (1981) in Biology of Carbohydrates (Ginsburg, V., ed) Vol. 1, pp. 199–314, Academic Press, New York
5. Piccon, C., Atiken, A., Bilham, T., and Cohen, P. (1982) Eur. J. Biochem. 124, 37–45
6. Roach, P. J., DePaoli-Roach, A. A., and Larner, J. (1978) J. Cyclic Nucleotide Res. 4, 245–257
7. DePaoli-Roach, A. A., Roach, P. J., and Larner, J. (1979) J. Biol. Chem. 254, 4212–4219
8. Embi, N., Rylatt, D. B., and Cohen, P. (1979) Eur. J. Biochem. 100, 339–347

*In fact, staining of polyacrylamide gels of CNBr fragments of liver and muscle glycogen synthases revealed differences in the fragmentation pattern extending much beyond those apparent from examination only of the phosphorylated CNBr fragments, as in Fig. 5.*

9. Walsh, K. X., Millikan, D. M., Schlenker, K. K., and Reimann, E. M. (1979) J. Biol. Chem. 254, 6611–6616
10. Soderling, T. R., Srivastava, A. K., Bass, M. A., and Khatri, B. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2536–2540
11. Soderling, T. R., Shearman, V. S., and Ericsson, L. H. (1979) FEBS Lett. 106, 181–186
12. Payne, M. E., and Soderling, T. R. (1980) J. Biol. Chem. 255, 8054–8056
13. Ahmad, Z., DePaoli-Roach, A. A., and Roach, P. J. (1982) J. Biol. Chem. 257, 8348–8355
14. Woodgett, J. R., Tonks, N. K., and Cohen, P. (1982) FEBS Lett. 148, 5–11
15. Itarte, E., and Huang, K.-P. (1980) J. Biol. Chem. 254, 4052–4057
16. Ahmad, Z., Camici, M., DePaoli-Roach, A. A., and Roach, F. J. (1984) J. Biol. Chem. 259, in press
17. DePaoli-Roach, A. A., Ahmad, Z., and Roach, P. J. (1981) J. Biol. Chem. 256, 8955–8962
18. Ahmad, Z., DePaoli-Roach, A. A., and Roach, P. J. (1982) J. Biol. Chem. 257, 5873–5876
19. Huang, K.-P., Itarte, E., Singh, T. J., and Akatsuka, A. (1982) J. Biol. Chem. 257, 3236–3242
20. Ma, J., and Traugh, J. A. (1982) Curr. Top. Cell. Regul. 21, 101–127
21. Hennings, B. A., Yellowles, D., Kernohan, J. C., and Cohen, P. (1981) Eur. J. Biochem. 119, 443–451
22. Yang, S.-D., Vandenheede, J. R., Goris, J., and Merlevede, W. (1980) J. Biol. Chem. 255, 11759–11767
23. Vandenheede, J. R., Yang, S.-D., Goris, J., and Merlevede, W. (1980) J. Biol. Chem. 255, 11768–11774
24. DePaoli-Roach, A. A., Roach, P. J., and Larner, J. (1979) J. Biol. Chem. 254, 12062–12068
25. Christmas, J.-L., Aylward, J. H., Shikama, H., and Exton, J. H. (1981) FEBS Lett. 127, 97–100
26. Sheorain, V. S., Khatra, B. S., and Soderling, T. R. (1981) FEBS Lett. 127, 94–96
27. Ubing, R. J., Shikama, H., and Exton, J. H. (1981) FEBS Lett. 134, 185–188
28. Sheorain, V. S., Khatra, B. S., and Soderling, T. R. (1982) J. Biol. Chem. 257, 3462–3470
29. Parker, P. J., Embi, N., Caudwell, B., and Cohen, P. (1982) Eur. J. Biochem. 124, 47–55
30. Parker, P. J., Caudwell, F. B., and Cohen, P. (1983) Eur. J. Biochem. 130, 227–234
31. Lawrence, J. C., Jr., Hiken, J. F., DePaoli-Roach, A. A., and Roach, P. J. (1983) J. Biol. Chem. 258, 10710–10719
32. Roach, P. J., DePaoli-Roach, A. A., Hiken, J., and Lawrence, J. C., Jr. (1982) Diabetes 31, 124A
33. Hems, D. A., and Whitten, P. D. (1980) Physiol. Rev. 60, 1–50
34. Exton, J. H. (1979) J. Cyclic Nucleotide Res. 5, 277–287
35. Camici, M., DePaoli-Roach, A. A., and Roach, P. J. (1982) J. Biol. Chem. 257, 9888–9901
36. Camici, M., DePaoli-Roach, A. A., and Roach, P. J. (1984) J. Biol. Chem. 259, in press
37. Takeda, Y., Brewer, H. B., Jr., and Larner, J. (1975) J. Biol. Chem. 251, 1913–1919
38. Kaji, T., Moriyama, K., Terada, T., and Ogawa, M. (1981) J. Biol. Chem. 256, 7601–7606
39. Smith, C. H., Villar-Palasi, C., Brown, N. E., Schlenker, K. K., Rosenberg, A. M., and Larner, J. (1972) Methods Enzymol. 28, 539–539
40. Davidson, M. M. (1980) J. Biol. Chem. 255, 8415–8422
41. Smirch, T. D., Jordan, J. E., and Exton, J. H. (1982) J. Biol. Chem. 257, 10798–10804
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