Phosphorylation and Inactivation of Liver Glycogen Synthase by Liver Protein Kinases*

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Michinori Imazu, W. Garrison Strickland‡, Ted D. Chrisman§, and John H. Exton†

From the Laboratories for the Studies of Metabolic Diseases, Howard Hughes Medical Institute, and Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

A rapid method for purifying glycogen synthase α from rat liver was developed and the enzyme was tested as a substrate for nine different protein kinases, six of which were isolated from rat liver. The enzyme was phosphorylated on a 17-kDa CNBr fragment to ~1 phosphate/87-kDa subunit by phosphorylase b kinase from muscle or liver with a decrease in the activity ratio (~Glc-6-P/+Glc-6-P) from 0.95 to 0.6. Calmodulin-dependent glycogen synthase kinase from rabbit liver produced a similar phosphorylation pattern, but a smaller activity change. The catalytic subunit of beef heart CAMP-dependent protein kinase incorporated >1 phosphate/subunit initially into a 17-kDa CNBr peptide and then into a 27–30-kDa CNBr peptide, with an activity ratio decrease to 0.5. Glycogen synthase kinases 3, 4, and 5 and casein kinase 1 were purified from rat liver. Glycogen synthase kinase 3 rapidly phosphorylated liver glycogen synthase to 1.5 phosphate/subunit with incorporation of phosphate into 3 CNBr peptides and a decrease in the activity ratio to 0.3. Glycogen synthase kinase 4 produced a pattern of phosphorylation and inactivation of liver synthase which was very similar to that caused by phosphorylase b kinase. Glycogen synthase kinase 5 incorporated 1 phosphate/subunit into a 24-kDa CNBr peptide, but did not alter the activity of the synthase. Casein kinase 1 phosphorylated and inactivated liver synthase with incorporation of phosphate into a 24-kDa CNBr peptide. This kinase and glycogen synthase kinase 4 were more active against muscle glycogen synthase. Calcium-phospholipid-dependent protein kinase from brain phosphorylated liver and muscle glycogen synthase on 17- and 27-kDa CNBr peptides, respectively. However, there was no change in the activity ratio of either enzyme.

The following conclusions are drawn. 1) Liver glycogen synthase α is subject to multiple site phosphorylation. 2) Phosphorylation of some sites does not per se control activity of the enzyme under the assay conditions used. 3) Liver contains most, if not all, of the protein kinases active on glycogen synthase previously identified in skeletal muscle.

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§ Senior Associate, Howard Hughes Medical Institute.

† Investigator, Howard Hughes Medical Institute. To whom reprint requests should be addressed.

Despite the importance of glycogen synthase in the regulation of liver glycogen metabolism, there have been very few studies of the phosphorylation and inactivation of purified liver glycogen synthase by protein kinases. This is in contrast to the situation with muscle glycogen synthase. In 1976, Killilea and Whelan [1] reported the purification of rabbit liver glycogen synthase and showed that it could be phosphorylated and inactivated by CAMP-dependent protein kinase. In 1979, Jett and Soderling [2] reported that rat liver glycogen synthase could be phosphorylated not only by CAMP-dependent protein kinase but also by a CAMP-independent protein kinase isolated from liver, and they correlated the incorporation of phosphate (up to 2–3 phosphate molecules/subunit) with inactivation of the enzyme by either kinase. Recently, Huang et al. [3] confirmed that CAMP-dependent protein kinase phosphorylated liver glycogen synthase, but were unable to demonstrate any decrease in the activity ratio (~Glc-6-P/+Glc-6-P). They also showed that casein kinase 1 from muscle catalyzed the incorporation of approximately 2 phosphate molecules/subunit into the liver enzyme, but with no detectable inactivation (5). Inactivation was only observed when casein kinase 1 was added following an initial incubation with CAMP-dependent protein kinase (3).

There have also been few reports of liver protein kinases active on glycogen synthase, and the majority of the studies have employed skeletal muscle glycogen synthase as substrate. The catalytic subunit of CAMP-dependent protein kinase (4) and phosphorylase b kinase (5) have been purified to homogeneity from liver. A Ca2+-calmodulin-dependent protein kinase with high specificity towards muscle glycogen synthase has also been isolated from this tissue (6–8). There are also at least two other CAMP-independent protein kinases in liver which act on glycogen synthase (2, 9–13). One of these (10, 11) which corresponds to glycogen synthase kinase 5 (PCo7 or casein kinase 2) of skeletal muscle (14–16) has been extensively phosphorylated from rabbit liver and shown to phosphorylate muscle glycogen synthase to the level of 6.5 phosphate/subunit with little inactivation (11). Another kinase purified from rat or rabbit liver (10, 12) corresponds to casein kinase 1 of muscle (17). It has been reported to phosphorylate muscle glycogen synthase to greater than 4 phosphates/subunit with substantial inactivation of the enzyme (10, 12). Other studies indicate the presence of additional CAMP-independent protein kinases in liver which act on glycogen synthase (2, 9, 13).

This report presents a procedure for purifying glycogen synthase from rat liver and also a study of the phosphorylation and inactivation of this enzyme by nine protein kinases, six of which were isolated from liver. It was undertaken as part of a program to define the protein kinases which regulate liver glycogen synthase in vivo.
**Experimental Procedures**

**Enzyme Assays—**Glycogen synthase activity was measured using a modification of the method of Thomas et al. (18). The enzyme was diluted in 50 mM Tris-HCl (pH 7.6), 27 mM EDTA, 50 mM NaF, and 1 mg/ml of bovine serum albumin. Reactions were initiated by adding 25 μl of the diluted enzyme to 25 μl of reaction mixture containing 10 mM [3H]UDP-glucose (0.3 μCi/mmole), 50 mM Tris-HCl (pH 7.6), 27 mM EDTA, 50 mM NaF, and 10% (w/v) oyster glycogen plus or minus 0.5 mM glucose-6-P. After 20 min at 30 °C, a 40-μl aliquot was spotted on filter paper (Whatman 3MM), and processed as described by Thomas et al. (18). One unit of activity is that which catalyzes the incorporation of 1 μmol of glucose from UDP-glucose into glycogen/min in the presence of glucose-6-P. The glycogen synthase activity ratio is defined as the activity in the absence of glucose-6-P divided by the activity in the presence of 5 mM glucose-6-P.

Protein kinase assays were conducted in 25 μl of a reaction mixture containing 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, and 0.1 mM ATP (200 to 2000 cpm/pmol). When skeletal muscle glycogen synthase was the substrate, it was 0.05 mg/ml, whereas casein was 2 mg/ml. After 10 min at 30 °C, a 20-μl aliquot was spotted on filter paper (Whatman 3MM), and further processed as described by Christman et al. (19). Controls lacking substrate or protein kinase were routinely run.

**Purification of Rat Liver Glycogen Synthase Kinase—**An outline of the procedures used in the purification of these kinases is given in Fig. 2. Most of these procedures have been adapted from the work of others as indicated. All steps were carried out at 0-4 °C. The 120,000 × g supernatant fraction prepared from 280 g of rat liver by the method of Chrisman et al. (5) was applied to a phosphocellulose column (0.6 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM NaCl, 20 mM 2-mercaptoethanol, and 10% of glycerol (Buffer A) at 80 ml/h. Three peaks of glycogen synthase-phosphorylating activity were eluted from the column (Fig. 3); the first at approximately 0.2 M NaCl, the second at approximately 0.4 M NaCl, and the third at approximately 0.6 M NaCl, coincident with the peak of casein kinase activity. By analogy with the enzymes isolated from skeletal muscle (14-16) or rabbit liver (11), the peaks of kinase activity eluted around 0.2 and 0.6 M NaCl were designated glycogen synthase kinase 3 and glycogen synthase kinase 5, respectively. Likewise, the activity eluted at 0.4 M NaCl was designated as a mixture of glycogen synthase kinase 4 (14, 15) and casein kinase 1 (17). The three major kinase fractions were pooled and dialyzed against Buffer A containing 50% glycerol and were stored at -20 °C until further use.

**Further Purification of Glycogen Synthase Kinase—**All buffers for chromatography contained 10% glycerol. The glycogen synthase kinase 3 fraction from the phosphocellulose column (0.2 M NaCl) was further purified by a modification of the method of Homans and Thomas et al. (20). This modified method utilized DE52, Affi-Gel blue, Sephadex G-100, and CM-Sephadex, respectively, with the glycogen synthase-agarose chromatography step being omitted. The major portion of the synthase applied to the DE52 column was in the "flowthrough" fraction. The bulk of the enzyme was applied to the CM-Sephadex (pH 7.5), 1 mM EDTA, 20 mM 2-mercaptoethanol, and 10% of glycerol (Buffer A) at 80 ml/h. Three peaks of glycogen synthase-phosphorylating activity were eluted from the column (Fig. 4); the first at 0.3 M NaCl, the second at 0.4 M NaCl, and the third at 0.5 M NaCl. The active fractions eluted at 0.3 M NaCl were further purified on Sephadex G-100. The apparent Mr of this enzyme was about 70,000 by this gel filtration. The Sephadex G-100 fraction was applied to an Affi-Gel blue column and eluted with Buffer A containing 50% glycerol. The Sephadex G-100 fraction was further chromatographed on DEAE-Sepharose (15), yield-
ine (Serdary Research Laboratories, Inc.) were mixed. After chloroform and hexane were removed in vacuo, the residue was suspended in 20 mM Tris-HCl (pH 7.5) by sonication and stored at -70 °C. Partially dephosphorylated casein was from Sigma. DEAE-Sepharose CL-6B, CM-Sephadex C-50, Sephadex G-100 (fine grade), and Concanavalin A-Sepharose were from Pharmacia. DEAE-cellulose (DE52) and phosphocellulose (P11) were from Whatman. Affi-Gel blue was from Bio-Rad. All other materials were from the sources described previously (35) or commercial sources.

RESULTS

Purification of Rat Liver Glycogen Synthase —Glycogen synthase a was purified from the rat liver glycogen pellet by a new rapid procedure. All steps were carried out at 0–4 °C unless stated otherwise. Washed glycogen pellets (5) were quickly frozen in liquid N₂ and stored at -70 °C until required. Frozen pellets (6.5 g) were suspended (Dounce homogenizer) in 2 volumes of 5 mM imidazole HCl (pH 7.2), 1 mM EDTA, 40 mM 2-mercaptoethanol, and 20% glycerol (Buffer B) containing 20 mM glucose and 2 µg/ml each of the protease inhibitors leupeptin, antipain, chymostatin, and pepstatin. The suspension was incubated at 25 °C for 30 min during which time the activity ratio of the glycogen synthase increased from 0.3 to 0.9. It was then mixed with 8 ml of DEAE-Sepharose, equilibrated with Buffer B, and stirred for 30 min. Under these conditions, most of the proteins except synthase were adsorbed to the resin. The resin was collected by filtration (sintered glass) and washed with approximately 15 ml of Buffer B until the filtrate was colorless. A 25-ml volume of the DEAE-Sepharose flow-through fraction was diluted with 105 ml of 10 mM Na β-glycerophosphate (pH 7.6), 1 mM EDTA, 40 mM 2-mercaptoethanol, 10% glycerol, and 0.25% oyster glycogen (Buffer C) and was applied at 30 ml/h to a DE52 column (2.6 × 16 cm) equilibrated with Buffer C in the absence of glycogen. The column was washed successively with 80 ml of Buffer C and 400 ml of 0.07 M NaCl in Buffer C. The glycogen synthase was then eluted with 0.25 M NaCl in Buffer C, and active fractions were pooled and dialyzed against 1 liter of 10 mM Na β-glycerophosphate (pH 7.6), 1 mM EDTA, 40 mM 2-mercaptoethanol, and 30% glycerol (Buffer D) for 3 h prior to rapid freezing in liquid N₂ and stored at -70 °C. The enzyme was stable to storage for at least 6 months.2

The purification scheme described could be accomplished, starting with the washed glycogen pellet, in about 24 h. It yielded an enzyme which was purified ~3000-fold from the 8700 × g rat liver supernatant fraction and ~18-fold from the washed glycogen pellet, with between 20 and 30% recovery (Table I). The final enzyme preparation exhibited a specific activity of 47,000 unit/mg and an activity ratio of 0.96 ± 0.02, and contained 0.2–0.5 alkali-labile phosphate/87,000 Mᵣ subunit. It showed one major band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1) the mobility of which corresponded to a subunit Mᵣ of 87,000 ± 2,000. The enzyme preparation contained no detectable protein kinase activity (see Figs. 4–12). The apparent Kᵣ for UDP-Glc was 0.6 mM (—Glc-6-P) and 0.3 mM (+5 mM Glic-6-P).

Phosphorylation and Inactivation of Liver Glycogen Synthase by CAMP-dependent Protein Kinase—As shown in Fig. 4, CAMP-dependent protein kinase catalyzed within 30 min a phosphorylation of liver glycogen synthase to the extent of 1 phosphate/87,000 Mᵣ subunit followed by a slower incorporation of a further 0.6 phosphate over the next 5 h under the conditions used i.e. substrate/kinase ratio. The activity ratio of the enzyme showed a corresponding biphasic change with a rapid decline from 0.95 to 0.68 then a slower fall to 0.46 (Fig. 4), and in the absence of kinase, there was no incorporation of 32P or inactivation of glycogen synthase (Fig. 4). These results indicate that the synthase preparation was free of contaminating kinases. Phosphorylation of the synthase to 1.3 phosphate/subunit resulted in a 3-fold increase in the Kᵣ for UDP-Glc in the absence of Glic-6-P.

To analyze the sites at which the synthase was phosphorylated, the synthase was prepared as previously (26).

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Purification Yield</th>
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<tr>
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<td>7344</td>
<td>0.016</td>
<td>100</td>
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<tr>
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<td>2.23</td>
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<td>DE52 Chromatography</td>
<td>33.0</td>
<td>0.68</td>
<td>48.6</td>
<td>3193</td>
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</table>

2 For some experiments, the enzyme was concentrated using concanavalin A-Sepharose. Active fractions from the DE52 column were applied to a concanavalin A-Sepharose column equilibrated with 10 mM Na β-glycerophosphate (pH 7.6), 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 40 mM 2-mercaptoethanol, and 10% glycerol (Buffer E). The column was then washed with 5 column volumes of Buffer E and glycogen synthase was eluted with 0.2 M glucose in Buffer E.
Phosphorylation and Inactivation of Liver Glycogen Synthase

**FIG. 3.** Phosphocellulose column chromatography of liver supernatant. The column flow rate was 80 ml/h and the elution schedule involved a wash (3000 ml) with 0.05 M NaCl in Buffer A, followed by a linear gradient (600 ml) formed from equal volumes of 0.05 M NaCl in Buffer A and 0.2 M NaCl in Buffer A. This was followed by 0.2 M NaCl in Buffer A (1800 ml), and finally by a linear gradient (2000 ml) formed from equal volumes of 0.2 M NaCl in Buffer A and 1.0 M NaCl in Buffer A. Fractions (8 ml) were assayed for glycogen synthase kinase (O-O), casein kinase (A-A), absorbance at 280 nm (O-O-O), and NaCl concentration (- - -).

**FIG. 4.** Phosphorylation and inactivation of liver glycogen synthase by the catalytic subunit of cAMP-dependent protein kinase (cAMP K). The reaction mixture was as described under "Experimental Procedures" except that the buffer was 20 mM triethanolamine HCl (pH 7.2), magnesium acetate was 2.5 mM and [γ-³²P] ATP was 0.2 mM. Liver glycogen synthase (23 µg/ml) was incubated with or without the catalytic subunit of the cAMP-dependent protein kinase (7.5 µg/ml) as indicated. Other details are given under "Experimental Procedures."

**FIG. 5.** Electrophoretic analysis of CNBr phosphopeptides from glycogen synthase phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (top) and by liver and muscle phosphorylase kinase (bottom). Top, the reaction mixture was as described in Fig. 4 except that bovine serum albumin was replaced by 0.5 mg/ml of human γ-globulin and glycogen was omitted. Exp. 1, A, protein kinase; B, liver synthase; C, liver synthase plus protein kinase; D, muscle synthase plus protein kinase; E, muscle synthase. When present, glycogen synthase was 40 µg/ml and protein kinase was 1.5 µg/ml in the 180-min phosphorylation incubation. Exp. 2, glycogen synthase (19 µg/ml) was incubated with the protein kinase (3 µg/ml) for 5 min (A and D), 30 min (B), and 240 min (C). A-C, with liver synthase; D, with muscle synthase. Aliquots were removed for fragmentation with CNBr, gel electrophoresis, and autoradiography as described under "Experimental Procedures." The numbers above each lane denote phosphate incorporated per subunit of synthase. Bottom, the reaction mixtures were as described in Fig. 6 except that when skeletal muscle phosphorylase kinase was used.

It is not known whether this represents a tissue or species difference or results from the different methods of enzyme purification employed.

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**Table:**

<table>
<thead>
<tr>
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<td>0.81</td>
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</tr>
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<td>C</td>
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<tr>
<td>D</td>
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<table>
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<tbody>
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<td>0.74</td>
<td>0.81</td>
<td>0.55</td>
</tr>
<tr>
<td>B</td>
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<td>0.68K</td>
<td>0.43K</td>
<td>0.30K</td>
</tr>
<tr>
<td>C</td>
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<td>0.20K</td>
<td>0.14K</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.00K</td>
<td>0.03K</td>
<td>0.07K</td>
<td></td>
</tr>
</tbody>
</table>

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The autoradiograph (Exp. 1, lane B) confirms the lack of contamin.
Phosphorylation and Inactivation of Liver Glycogen Synthase

Phosphorylation and inactivation of liver glycogen synthase by the enzyme

Fig. 6 illustrates that phosphorylase b kinase from rat liver (left) and rabbit skeletal muscle (right) incorporated approximately 0.9 phosphate/subunit into liver glycogen synthase with a decrease in the activity ratio to 0.5. The phosphorylation and inactivation of liver glycogen synthase by the enzyme from muscle were respectively inhibited 90 and 85% by EGTA. EGTA produced less complete inhibition of the effects of liver phosphorylase b kinase on liver glycogen synthase.

As shown in Fig. 5 (bottom), liver or muscle phosphorylase b kinase promoted the incorporation of \( ^{32}P \) from \( [\gamma-^{32}P]ATP \) almost exclusively into the \( M_1 \), 17,000 CNBr fragment of liver glycogen synthase. With muscle glycogen synthase, both kinases phosphorylated predominantly the \( M_1 \), 12,000 CNBr peptide (Fig. 5, bottom).

Effect of Liver Calmodulin-dependent Glycogen Synthase Kinase—The calmodulin-dependent glycogen synthase kinase of liver has been postulated to play a major role in the regulation of liver glycogen synthase by calcium-dependent hormones (6, 8). However, muscle glycogen synthase has been used as a substrate in all previously published studies of this enzyme (6-8). As shown in Fig. 7 (left), this kinase phosphorylated liver glycogen synthase to a level of about 1 phosphate/subunit, with minimal phosphorylation in the absence of calmodulin or Ca\(^{2+}\). However, the decrease in activity ratio caused by this phosphorylation was only 0.2-0.3 which is less than that caused by a similar degree of phosphorylation by either liver or muscle phosphorylase b kinase (c.f. Fig. 6). Gel electrophoresis in the presence of sodium dodecyl sulfate of the CNBr fragments of the \( ^{32}P \)-labeled enzyme showed \( ^{32}P \) in the \( M_1 \), 17,000 fragments (Fig. 8, top). As expected, the effects of the kinase were abolished by EGTA (Fig. 7) and trifluoperazine (data not shown).

For comparison, the above studies were repeated with the same concentration of glycogen synthase from rabbit skeletal muscle. Fig. 7 (right) shows that this glycogen synthase was phosphorylated to a level of 1 phosphate/subunit slightly more rapidly than the liver enzyme. However, the accompanying decrease in activity ratio (0.3-0.4) was greater than that observed for the liver enzyme. The CNBr fragments of the \( ^{32}P \)-labeled muscle enzyme showed \( ^{32}P \) in both \( M_1 \), 27,000 and 12,000 peptides (Fig. 8, top).

Effects of Liver Glycogen Synthase Kinases 3, 4, and 5 and Casein Kinase I—As described in detail under “Experimental Procedures,” enzymes corresponding to skeletal muscle glycogen synthase kinases 3, 4, and 5 (14-16) were purified from rat liver using phosphocellulose chromatography and other procedures. Fig. 9 shows the phosphorylation of liver glycogen synthase by liver glycogen synthase kinases 3 and 5. Synthase kinase 3 was very active on this substrate (right) and rapidly phosphorylated it to the extent of 1.5 phosphate/subunit with a decrease in the activity ratio to about 0.3. With time, slightly more phosphate was incorporated and the activity ratio declined to less than 0.2. Similar findings were obtained with muscle glycogen synthase as substrate (data not shown).

When liver synthase kinase 5 was employed (Fig. 9, left), there was a slower incorporation of 1 phosphate/subunit over 5 h. However, there was no detectable change in the activity ratio under the assay conditions used. Similar findings were obtained using muscle glycogen synthase as substrate (data not shown). PKI was without effect on the actions of synthase kinases 3 and 5 indicating that they were not contaminated.

bovine serum albumin was replaced by 9.5 mg/ml of human IgG. A, liver phosphorylase kinase; B, liver synthase plus liver phosphorylase kinase; C, muscle synthase plus liver phosphorylase kinase; D, muscle phosphorylase kinase; E, liver synthase plus muscle phosphorylase kinase; F, muscle synthase plus muscle phosphorylase kinase; G and I, liver synthase; H and J, muscle synthase. The reaction mixture (G and H) was for liver phosphorylase kinase, and I and J were for muscle phosphorylase kinase. When present, glycogen synthase was 40 \( \mu \)g/ml, liver phosphorylase kinase was 4.6 \( \mu \)g/ml, and muscle phosphorylase kinase was 2 \( \mu \)g/ml in the 100-min phosphorylation incubation.

FIG. 6. Phosphorylation and inactivation of liver glycogen synthase by skeletal muscle or liver phosphorylase kinase (Ph K).

The reaction mixture was as described under “Experimental Procedures,” except that when liver phosphorylase kinase was used, the buffer was 20 mM Tris-HCl (pH 7.5) and \( [\gamma-^{32}P]ATP \) was 0.5 mM, and when muscle phosphorylase kinase was used, the buffer was 20 mM Tris-HCl (pH 8.6), magnesium acetate was 4 mM, and \( [\gamma-^{32}P]ATP \) was 0.5 mM in the presence of 19 pg/ml of calmodulin. Liver synthase (19 \( \mu \)g/ml) was incubated alone or with skeletal muscle phosphorylase kinase (23 \( \mu \)g/ml) (left) or with liver phosphorylase kinase (23 \( \mu \)g/ml) (right) as indicated. To overcome interference due to autophosphorylation of phosphorylase kinase, the measurement of \( ^{32}P \) incorporation into glycogen synthase was carried out using gel electrophoresis as described under “Experimental Procedures.”
Fig. 8. Electrophoretic analysis of CNBr phosphopeptides from glycogen synthase phosphorylated by calmodulin-dependent glycogen synthase kinase (top) or liver glycogen synthase kinases 3 and 5 (bottom). Top, the reaction mixture was prepared as described in Fig. 7 except that glycogen was omitted. A, calmodulin-dependent synthase kinase; B–D, liver synthase plus synthase kinase; E, muscle synthase plus synthase kinase; F, liver synthase; G, muscle synthase; A, B, E, F, and G, in the presence of 0.2 mM CaCl₂ and 10 µg/ml of calmodulin; C, in the presence of 0.2 mM CaCl₂; D, in the presence of 0.5 mM EGTA. When present, glycogen synthase was 40 µg/ml and synthase kinase was 2.4 µg/ml in the 180-min phosphorylation incubation. The numbers above each lane denote phosphate incorporated per subunit of synthase. Bottom, the reaction mixture was as described in Fig. 9 except that glucagon was omitted. Exp. 1, liver glycogen synthase (19 µg/ml) was incubated with glycogen synthase kinase 3 (11 µg/ml) for 90 min. Exp. 2, A, glycogen synthase kinase 3, B, liver synthase plus glycogen synthase kinase 3; C, muscle synthase plus glycogen synthase kinase 3; D, glycogen synthase kinase 5; E, liver synthase plus glycogen synthase kinase 5; F, muscle synthase plus glycogen synthase kinase 5; G, liver synthase; H, muscle synthase. When present, glycogen synthase was 40 µg/ml, glycogen synthase kinase 3 was 11 µg/ml, and glycogen synthase kinase 5 was 1.8 µg/ml in the 180-min phosphorylation reaction.

with cAMP-dependent protein kinase.

Fig. 8 (bottom) shows the labeling pattern of the CNBr fragments of the liver enzyme prepared by incubating it with [γ³²P]ATP and synthase kinases 3 and 5. With synthase kinase 3, there was heavy labeling of M₈, 2, 000, M₉, 27, 000, and M₁₀, 22, 000 peptides, but also ³²P in the M₁₀, 17, 000 fragment. The pattern was different with synthase kinase 5, i.e. there was labeling only of a M₁₀, 24, 000 peptide. Similar findings were obtained using muscle glycogen synthase as substrate (Fig. 8, bottom).

Fig. 10 (left) shows that liver glycogen synthase a was phosphorylated by rat liver glycogen synthase kinase 4. With the incorporation of approximately 1.1 phosphate/subunit, there was a decrease in the activity ratio to 0.44. The enzyme was more active towards muscle glycogen synthase causing approximately 1.5 phosphate/subunit to be incorporated with almost complete inactivation. The actions of synthase kinase 4 were unaffected to PKI indicating its lack of contamination with cAMP-dependent protein kinase. Autoradiography of CNBr fragments of the ³²P-labeled synthases from liver and muscle (Fig. 11, top) showed that the isotope was incorporated

4 This amount of PKI inhibited 3 µg of the catalytic subunit of cAMP-dependent protein kinase on the phosphorylation of liver glycogen synthase.

5 It is recognized that some of these bands may represent the same peptide, but phosphorylated to different degrees and therefore migrating differently during electrophoresis.

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**Fig. 9.** Phosphorylation and inactivation of liver glycogen synthase by liver glycogen synthase kinases 3 and 5. The reaction mixture was as described under “Experimental Procedures.” Liver synthase (19 µg/ml) was incubated with liver glycogen synthase kinase 3 (GSK3) (11 µg/ml) (left), or liver glycogen synthase kinase 5 (GSK5) (3.5 µg/ml) (right), or without synthase kinase as indicated.

**Fig. 10.** Phosphorylation and inactivation of liver glycogen synthase by liver glycogen synthase kinase 4 or liver casein kinase 1. The reaction mixture was as described under “Experimental Procedures.” Liver synthase (19 µg/ml) was incubated with liver glycogen synthase kinase 4 (GSK4) (12 µg/ml) (left), liver casein kinase 1 (7.2 µg/ml) (right), or without kinase as indicated.
Phosphorylation and Inactivation of Liver Glycogen Synthase

Fig. 11. Electrophoretic analysis of CNBr phosphopeptides from glycogen synthase phosphorylated by glycogen synthase 4 or casein kinase 1 (top) or calcium-phospholipid-dependent protein kinase (bottom). Top, the reaction mixture was as described in Fig. 10 except that glycogen was omitted. When present, glycogen synthase was 40 μg/ml and incubation time was 180 min. Exp. 1, A, glycogen synthase kinase 4; B, liver synthase plus glycogen synthase kinase 4; C, muscle synthase plus glycogen synthase kinase 4; D, liver synthase; E, muscle synthase. Glycogen synthase kinase 4 was 12 μg/ml. Exp. 2, A, casein kinase 1; B, liver synthase plus casein kinase 1; C, muscle synthase plus casein kinase 1; D, liver synthase; E, muscle synthase. Casein kinase 1 was 7.2 μg/ml. The numbers above each lane denote phosphate incorporated/subunit of glycogen synthase. Bottom, the reaction mixture was as described in Fig. 12 except that bovine serum albumin was replaced by 0.5 mg/ml of human γ-globulin and glycogen was omitted. A, calcium-phospholipid-dependent protein kinase; B and C, liver synthase plus protein kinase; D, muscle synthase plus protein kinase; E, liver synthase; F, muscle synthase; A, B, D, E, and F, in the presence of 0.5 mM CaCl2, 0.8 μg/ml of diolein, and 40 μg/ml of phosphatidylycerine; C, in the presence of 0.5 mM EGTA. When present, glycogen synthase was 40 μg/ml and calcium-phospholipid-dependent protein kinase was 3.8 μg/ml in the 100-min phosphorylation incubation.

mainly into the M, 17,000 and M, 12,000 peptides, respectively.

Liver synthase was also phosphorylated and inactivated by casein kinase 1 from liver (Fig. 10, right). Analysis of the distribution of 32P in the CNBr fragments of the labeled enzyme showed radioactivity exclusively in a M, 24,000 peptide. The same labeling pattern was seen with muscle glycogen synthase (Fig. 11, top), but this was a better substrate for the enzyme, showing the incorporation of approximately 0.9 phosphate/subunit and a decline in the activity ratio to 0.3.

Effect of Calcium-Phospholipid-dependent Protein Kinase—The other Ca<sup>2+</sup>-dependent enzyme which was tested on liver glycogen synthase was calcium-phospholipid-dependent protein kinase. Although this kinase is present in liver (36), it was purified from rat brain (23) for these studies. As shown in Fig. 12 (left), this kinase incorporated 32P into liver glycogen synthase in a Ca<sup>2+</sup>-dependent manner. However, the phosphorylation was not associated with inactivation of the enzyme. For comparison are shown the effects of the kinase on muscle glycogen synthase (Fig. 12, right). Again, there was phosphorylation of the synthase, but no change in its activity. Fig. 11 (bottom) shows the labeling pattern in the CNBr fragments of the liver and muscle enzyme. Incorporation of 32P was almost exclusively in the M, 17,000 peptide of the liver enzyme, however, mainly in the M, 27,000 peptide of the muscle enzyme.

DISCUSSION

Purification of Rat Liver Glycogen Synthase a—The scheme for purifying glycogen synthase a from rat liver described in this paper offer advantages over earlier schemes. Firstly, it is more rapid, allowing the enzyme to be purified from the liver glycogen pellet within 24 h. Secondly, it yields an enzyme which has a subunit M<sub>r</sub> of 87,000 ± 2,000 and is very little contaminated with lower M<sub>r</sub> forms (Fig. 1). Thirdly, it yields an enzyme with a high specific activity (47 ± 4 units/mg) and a high activity ratio (0.96 ± 0.02). Fourthly, the enzyme preparation is not detectably contaminated with protein kinases. Some preparations are detectably contaminated with trace amounts of phosphatase activities as determined by the release of 32P from liver synthase phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. However, no significant differences were detected between preparations containing phosphatase and those not containing.
Phosphorylation and Inactivation of Liver Glycogen Synthase

Phosphorylation and Inactivation of Liver Glycogen Synthase by CaM-dependent Protein Kinase—The present findings confirm the earlier findings of Killilea and Whelan (1) and Jett and Soderling (2) showing that CaM-dependent phosphorylation of liver glycogen synthase is associated with inactivation of the enzyme. They are in accord with numerous experiments showing that glucagon inactivates glycogen synthase in intact liver (e.g. Ref. 38) and that CaM inactivates the enzyme in broken cell liver preparations in the presence of MgATP (35). The failure of Huang et al. (3) to show inactivation of liver glycogen synthase by CaM-dependent protein kinase is probably due to the synthase preparation used, which showed two bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to M, 85,000 and 80,000. As stated by these workers and in accord with Camici et al. (37), this pattern is apparently the result of proteolysis. If proteolysis prevented phosphorylation of the sites which control activity, this could explain the relatively low extent of phosphorylation (0.7–1.0 phosphate/subunit) and the absence of an activity change reported by Huang et al. (3).

As illustrated in Figs. 4 and 5, phosphorylation by CaM-dependent protein kinase can exceed 1 phosphate/subunit and occurs on both the 27–30- and 17-kDa CNBr fragments indicating that it involves at least 2 sites. The 27–30- and 17-kDa fragments appear to correspond to the CB2 and CB1 peptides derived from the muscle enzyme (39). In contrast to the situation with muscle glycogen synthase, phosphorylation occurs initially (up to 30 min) on the 17-kDa fragment (Fig. 5). Since this is associated with inactivation (Fig. 4), it may be concluded that phosphorylation of a site(s) on this fragment of the liver enzyme controls its activity.

Phosphorylation and Inactivation of Liver Glycogen Synthase by Phosphorylase Kinases and Calmodulin-dependent Glycogen Synthase Kinase—The present data illustrate that liver glycogen synthase is a substrate for both liver and muscle phosphorylase b kinases, although the rate of phosphorylation is much less than with phosphorylase as substrate (5). Phosphorylation does not exceed 1 phosphate/subunit and occurs exclusively on the 17-kDa CNBr fragment corresponding to CB1 (Figs. 5 and 6). It causes a decrease in the activity ratio of 0.5 confirming the above conclusion that a site(s) on this part of the enzyme controls its activity.

Although it has been claimed that calmodulin-dependent glycogen synthase kinase may be important in the regulation of liver glycogen synthase by CaM-dependent hormones (6, 8, 40), there have been no previous demonstrations that this kinase can inactivate the liver enzyme. Figs. 7 and 8 illustrate that liver glycogen synthase is phosphorylated by the calmodulin-dependent kinase on the 17-kDa CNBr fragment. However, the activity change associated with this phosphorylation is less than that induced by this kinase in muscle glycogen synthase or induced in liver glycogen synthase by muscle or liver phosphorylase b kinase. Calmodulin-dependent glycogen synthase kinase phosphorylated muscle glycogen synthase on both 27- and 12-kDa CNBr peptides corresponding to the CB2 and CB1 peptides of Picton et al. (39).

In rat liver, calmodulin-dependent glycogen synthase kinase may be of minor importance in the CaM-dependent regulation of glycogen synthase since calmodulin or calmodulin antagonists fail to affect the activity of glycogen synthase in rat liver filtrates (35) or isolated hepatocytes (41). However, the enzyme may be important in other species e.g. rabbit where it is present in higher activity (42).

Phosphorylation and Inactivation of Liver Glycogen Synthase by Liver Glycogen Synthase Kinases 3, 4, and 5 and Casein Kinase 1—The present paper describes for the first time the purification from rat liver of enzymes corresponding to skeletal muscle glycogen synthase kinases 3 and 4 which phosphorylate liver and muscle glycogen synthase. As found previously for the skeletal muscle enzyme (43), rat liver glycogen synthase kinase 3 produced almost complete inactivation of liver or muscle glycogen synthase, as compared to the incomplete inactivation of liver glycogen synthase by other liver kinases. It thus possibly represents the major, CaM- and CaM2-independent glycogen synthase kinase activity in liver. Like its counterpart in skeletal muscle (39), it mainly phosphorylated sites on a 22–36-kDa CNBr peptide(s). Glycogen synthase kinase 4 from liver resembled phosphorylase b kinase with respect to its degree of phosphorylation and inactivation of liver glycogen synthase, but was more active toward the muscle enzyme. Like its equivalent in muscle (13, 39), the kinase phosphorylated mainly a small M, CNBr peptide of the liver or muscle glycogen synthase. Juhl et al. (13) have reported that glycogen synthase kinase 4 partially purified from rabbit liver phosphorylates muscle glycogen synthase specifically at site 2, which is located in the small M, CNBr peptide (39).

Glycogen synthase kinase 5 (casein kinase 2) has been purified previously from liver (10, 11) and shown to phosphorylate muscle glycogen synthase. As illustrated in Figs. 8 and 9, the liver kinase phosphorylates liver glycogen synthase on a 24-kDa CNBr peptide, but causes no alteration in its activity. Similar findings were obtained in the present study using muscle glycogen synthase as substrate, in agreement with Ahmad et al. (11) but not with Itarte et al. (10). Thus, glycogen synthase kinase 5 from liver has very similar catalytic properties to the enzyme from skeletal muscle (15, 16, 39, 44). The possibility that phosphorylation of liver glycogen synthase by glycogen synthase kinase 5 might alter its phosphorylation by glycogen synthase kinase 3 (45) was not tested.

Casein kinase 1 from liver was weakly active towards liver glycogen synthase (Fig. 10), but very active towards muscle glycogen synthase with respect to phosphorylation and inactivation, as observed previously by Itarte et al. (10) and Ahmad et al. (12). Casein kinase 1 from skeletal muscle has been reported to phosphorylate liver glycogen synthase to about 2 phosphate molecules/subunit without a change in activity unless the enzyme is prior phosphorylated with CaM-dependent protein kinase (3). However, since in this particular report, CaM-dependent protein kinase added alone did not inactivate the enzyme, the role of casein kinase 1 in the regulation of liver glycogen synthase remains uncertain.

Phosphorylation of Liver Glycogen Synthase by Calcium-Phospholipid-dependent Protein Kinase—Another kinase which could potentially be involved in mediating the effects
of Ca\textsuperscript{2+}-dependent hormones on glycogen synthase in liver is the calcium-phospholipid-dependent protein kinase discovered by Inoue et al. (46) and Takai et al. (47) and present in liver (36). As shown in Fig. 12, preparations of this enzyme from brain phosphorylate both liver and muscle glycogen synthase but cause no inactivation of these enzymes. Takai et al. (47) have also stated that the nonproteolytically activated kinase acts on the muscle enzyme, but have not indicated whether or not it causes inactivation.\textsuperscript{9} Although the kinase incorporated phosphate into the small (17-kDa) CNBr fragment of liver synthase, the site(s) phosphorylated must be different from that (those) phosphorylated by phosphorylase kinase, calmodulin-dependent synthase kinase, or glycogen synthase kinase 4 since these kinases cause inactivation. Surprisingly, the site(s) also appears to be different from that on muscle glycogen synthase which is located on the 27-kDa or CB2 peptide (Fig. 11, bottom).

Conclusions—The present findings indicate that liver glycogen synthase undergoes multisite phosphorylation like its counterpart in skeletal muscle. They also show that there is a general similarity between the phosphorylation sites in the two enzymes as revealed by CNBr peptide analysis, although CB1 from the muscle enzyme has a lower apparent M, than that from the liver enzyme. There is also a similarity in the activation in response to the two kinases by the two enzymes, with the muscle enzyme sometimes exhibiting greater inactivation for the same degree of phosphorylation. Another important point to emerge from the study is that the liver contains enzymes equivalent to most, if not all, of the skeletal muscle protein kinases active on glycogen synthase.

The function of cAMP-dependent protein kinase in the regulation of glycogen synthase in liver by glucagon or \(	extbeta\)-adrenergic agonists appears clear, but the kinases mediating the effects of Ca\textsuperscript{2+}-dependent hormones (\(\alpha\)-adrenergic agonists, vasopressin) on liver glycogen synthase is still in dispute. Glycogen synthase kinase 5 and Ca\textsuperscript{2+}-phospholipid-dependent protein kinase phosphorylate liver glycogen synthase, but do not alter its activity per se. However, they could influence its phosphorylation by other kinases. Glycogen synthase kinases 3 and 4 and casein kinase 1 inactivate liver glycogen synthase, but there are presently no known physiological regulators of these kinases in liver or muscle. Thus, clarification of the precise roles of these various kinases in the regulation of liver glycogen synthase and of their possible interactions requires much further work. The sites phosphorylated by the kinases also need to be defined by amino acid sequencing of phosphorylated tryptic peptides.

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\textsuperscript{9} Earlier reports (48, 49) showed that the protease-activated kinase both phosphorylated and inactivated muscle glycogen synthase.
Phosphorylation and inactivation of liver glycogen synthase by liver protein kinases.
M Imazu, W G Strickland, T D Chrisman and J H Exton


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