Purification and Characterization of Rabbit Liver Calmodulin-dependent Glycogen Synthase Kinase

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A rabbit liver CAMP-independent glycogen synthase kinase has been purified 4500-fold to a specific activity of 2.23 μmol per min per mg of protein using ion exchange chromatography on DEAE-Sephadex and phosphocellulose, gel filtration chromatography on Sepharose 6B, and affinity chromatography on calmodulin-Sepharose. This synthase kinase, which was completely dependent on the presence of calmodulin (apparent Kₐ = 0.1 μM) and calcium for activity, also catalyzed the phosphorylation of purified smooth muscle myosin light chain but not of smooth muscle myosin. Using 0.5 mm ATP, a maximal rate of phosphorylation of glycogen synthase was achieved in the presence of 10 mm magnesium acetate with a pH optimum of 7.8. Gel filtration experiments indicated a Stokes radius of about 70 Å and sucrose density gradient centrifugation data gave a sedimentation coefficient of 10.8 S. A molecular weight of approximately 300,000 was calculated. A definitive subunit structure was not determined, but major bands observed on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate corresponded to a doublet at 50,000 to 53,000. The calmodulin-dependent glycogen synthase kinase incorporated about 1 mol of ³²P per mol of synthase subunit into sites 2 and 1b associated with a decrease in the synthase activity ratio from 0.8 to about 0.4.

The calmodulin-dependent glycogen synthase kinase may mediate the effects of α-adrenergic agonists, vasopressin, and angiotsin II on glycogen synthase in liver.

The activity of glycogen synthase, which catalyzes the rate-limiting step in the biosynthesis of glycogen, is regulated in the cell both by allosteric modifiers and by covalent phosphorylation-dephosphorylation. Each 85,000- to 90,000-dalton subunit of glycogen synthase contains several phosphorylation sites which give rise to multiple forms of glycogen synthase. Phosphorylation and inactivation of the physiologically active a form of glycogen synthase to the inactive b form is a complex reaction involving at least three different classes of protein kinases: cyclic nucleotide-dependent, calcium-dependent, and cyclic nucleotide- and calcium-independent synthase kinase (for reviews see Refs. 1 and 2).

Cyclic AMP-dependent protein kinase can catalyze the incorporation of up to 3 mol of phosphate per mol of synthase subunit. Two mol are located in the COOH-terminal region (sites 1a and 1b) and 1 mol in the NH₂-terminal domain or site 2 (3-5). Several investigators have reported the partial purification, primarily from skeletal muscle, of a class of protein kinases which phosphorylate and inactivate glycogen synthase in a manner independent of cAMP (6-9). These kinases are also not affected by added calcium, EGTA, or calmodulin. Recent studies suggest that there may be several different CAMP-independent glycogen synthase kinases; however, their physiological function(s) remain(s) unknown. In general, these kinases seem to phosphorylate either site 2 or 3 (3, 5). Phosphorylase kinase, a calmodulin-containing enzyme, from liver or skeletal muscle catalyzes the incorporation into site 2 of 0.5-0.7 mol of P per mol of synthase subunit with partial inactivation of the synthase (10, 11).

In rat liver, α-adrenergic agonists, vasopressin, and angiotensin II have been shown to promote the inactivation of glycogen synthase, presumably by a calcium-mediated process (12). Furthermore, Garrison et al. (13) reported that treatment of isolated hepatocytes with vasopressin or angiotensin II led to a calcium-dependent increase in the phosphorylation of glycogen synthase. We therefore became interested in purifying calcium-dependent synthase kinases from liver.

We reported the existence of liver of a calcium, calmodulin-dependent protein kinase distinct from phosphorylase kinase which appears to be specific for glycogen synthase (14-15). This paper describes the extensive purification and some of the characteristics of this enzyme.

EXPERIMENTAL PROCEDURES

Materials

The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N’,N’-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; CaM, calmodulin.

1 Portions of this paper (including “Experimental Procedures,” some of the “Results,” Figs. 1-4, and Table I) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 This investigation was supported by National Institutes of Health Grant AM 17808. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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**Methods**

The glycogen synthase activity ratio (−glucose-6-P/+glucose-6-P) is defined as the ratio of activities determined in the absence and presence of glucose-6-P.

EGTA was included in the buffer during the initial steps in order to dissociate the calmodulin-dependent glycogen synthase kinase from calmodulin and to prevent calcium-dependent proteolysis.

**Chemical and Physical Properties**

The effect of pH on the phosphorylation of glycogen synthase by calmodulin-dependent glycogen synthase kinase was studied using several different buffers: Mes (pKₐ = 6.15), Pipes (pKₐ = 6.80), Hepes (pKₐ = 7.55), and glycylglycine (pKₐ = 8.40) (Fig. 5). The pH optimum for the phosphorylation was around 7.8. In many instances, individual buffers rather dramatically affected the glycogen synthase kinase activity. For example, at pH 7.0, calmodulin-dependent synthase kinase activity was over 3-fold higher using Hepes buffer rather than Pipes buffer. Moreover, the kinase activity at pH 8.0 was about 2-fold higher using Hepes rather than Tris (not shown). Consequently, a buffer combination of Mes, Heps, and glycylglycine was used.

**Calmodulin-dependent glycogen synthase kinase was purified from rabbit liver as described in the Miniprint.** The enzyme eluted from the calmodulin-Sepharose column with EGTA. The absorbance at 280 nm was less than 0.05. After pooling the calmodulin-eluted fractions of buffer A, the protein was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, and 0.1 mM EGTA. The protein was further dialyzed against 50 mM Mes, pH 6.0, and the conductivity was determined. The assays for calmodulin-dependent glycogen synthase kinase were linear with time and with all buffers.

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The inset of Fig. 5 illustrates the effect of pH (using the buffer combination) on phosphorylation of glycogen synthase by the calmodulin-dependent kinase confirming that the pH optimum was about pH 7.8.

The maximal rate of phosphorylation was achieved using a final concentration of 10 mM magnesium acetate and 0.5 mM ATP. Preliminary studies suggest a strong dependence on Mg²⁺ for activity and that neither 10 mM MnCl₂ nor 10 mM CaCl₂ could substitute for magnesium (Fig. 6).

Upon sucrose density gradient centrifugation, the calmodulin-dependent glycogen synthase kinase migrated as a single, symmetrical peak with a sedimentation coefficient of 10.6 S ± 0.1 (Fig. 7). The kinase activity toward glycogen synthase was completely dependent on calmodulin. Data obtained from gel filtration experiments on a calibrated Sepharose 6B column indicated a Stokes radius of 70 Å (Fig. 3). Based upon the sedimentation coefficient, the Stokes radius, and an assumed partial specific volume of 0.725 cm³/g (30), an approximate molecular weight of the calmodulin-dependent glycogen synthase kinase was calculated to be 300,000. A value of 1.57 was determined for the frictional ratio from the calculated molecular weight, the sedimentation coefficient, and the assumed partial specific volume.

The subunit composition of this enzyme is currently being investigated. Two major protein bands were resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with molecular weights of about 54,000 and 50,000 (Fig. 8). In some preparations, a faint band was observed at 19,000.

**Phosphorylation of Myosin Light Chain**—The CaM-dependent synthase kinase does not phosphorylate myosin light chain (M₉ = 20,000) isolated from either cardiac or skeletal muscle (14). Because the myosin light chain from smooth muscle has a different sequence around the phosphorylation site (Table 1) and is a substrate for other kinases (31), we tested it as a substrate for our kinase.

![Figure 5](http://www.jbc.org/)

**Table 1**

| Fraction | Total Protein (mg) | Total Activity (μmol/min/mg) | Specific Activity (μmol/min/mg) | Yield (%) | Purification Factor (F)
|----------|------------------|-----------------------------|-------------------------------|-----------|------------------------
| Supernatant | 7.607 | 7.48 | 4.9 x 10⁻⁴ | 100 | 1.0 |
| 10,000 g x Supernatant | 7.607 | 7.48 | 4.9 x 10⁻⁴ | 100 | 1.0 |
| 100,000 g x Supernatant | 8.108 | 8.17 | 4.5 x 10⁻⁴ | 95 | 0.9 |
| After DEAE Sephadex | 9.24 | 9.24 | 1.0 | 99 | 10 |
| After Phosphocellulose | 10 | 10 | 1.0 | 99 | 10 |
| After Sephadex 6B | 3 | 3 | 1.0 | 99 | 10 |
| After Cal Sephadex | 0.02 | 0.02 | 1.0 | 99 | 10 |

The inset of Fig. 5 depicts calmodulin-dependent glycogen synthase kinase activity (Fig. 8). The assays for kinase activity were not linear with respect to Mg. Incorporation into glycogen synthase

![Figure 4](http://www.jbc.org/)

![Figure 6](http://www.jbc.org/)
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Fig. 6. Divalent cation requirements. The calmodulin-dependent synthase kinase activity was assayed under standard conditions varying the magnesium acetate concentration as shown. In B, the assay was performed using either 10 mM magnesium, calcium, or manganese.

Fig. 7. Sucrose density gradient centrifugation of calmodulin-dependent glycogen synthase kinase. Calmodulin-dependent glycogen synthase kinase was purified through gel filtration on Sepharose 6B, rechromatographed on Sepharose 6B, and then concentrated by vacuum dialysis against buffer B. Two hundred µl of this sample was layered on 12 ml of a 5-20% sucrose density gradient in 50 mM Hepes, 1 mM dithiothreitol, pH 7.5 at 5 °C. Centrifugation was performed in a Beckman L5-65 ultracentrifuge using a Beckman SW 41 rotor (rmax = 109.5 mm) at 38,000 rpm for 18 h at 5 °C. Fractions (0.38 ml) were collected and assayed undiluted for calmodulin-dependent glycogen synthase kinase activity (A, left). The sedimentation coefficient was estimated relative to standard protein phosphorylase b (8.4 S), catalase (17.4 S), and glycogen synthase (13.3 S). Vc/Vr is the relative migration of each protein.

smooth muscle light chain was quite a good substrate with initial rates about 40-50% of those obtained with synthase as substrate (Table III). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that all the radioactivity was in the Mr = 20,000 myosin light chain (not shown). However, when smooth muscle myosin was tested as substrate, there was no phosphorylation of the endogenous light chain (Table III). In these experiments, we again confirmed that the myosin light chains from cardiac and skeletal muscle were not phosphorylated at a significant rate (not shown). Also, smooth muscle light chain kinase did not phosphorylate synthase in confirmation of earlier work (32).

Glycogen Synthase Phosphorylation Site Specificity—We have previously reported that the calmodulin-dependent synthase kinase can rapidly phosphorylate site 2 in glycogen synthase (14, 15). Synthase was phosphorylated to 0.9 mol of 32P/mol of subunit and subjected to CNBr cleavage followed by disc gel electrophoresis in the presence of sodium dodecyl sulfate. Approximately 40% of the 32P was in cyanogen bromide peptide 1 and 60% in cyanogen bromide peptide 2. In other experiments, synthase containing about 1 mol of 32P/subunit was subjected to tryptic digestion. The tryptic 32P peptides were analyzed by peptide mapping on reverse phase high performance liquid chromatography (Fig. 9). The peak eluting at 70 min corresponds to site 1b and the peak at 116 min corresponds to site 2 (5). The distribution of 32P between sites 1b and 2 was 34 and 66%, respectively.

Fig. 8. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of purified calmodulin-dependent glycogen synthase kinase. Purified calmodulin-dependent glycogen synthase kinase (2 µg) was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and stained using silver stain as described under "Experimental Procedures."

| Protein                | Substrate for CaM-dependent synthase kinase | Sequence | Reference |
|------------------------|------------------------------------------|----------|-----------|
| Smooth muscle light chain | Yes                                     | -R-A-T-S(P)-N-V-F-A- | 43       |
| Synthase site 2        | Yes                                     | -R-T-L-S(P)-V-S-S-L- | 11       |
| Synthase site 1b       | Yes                                     | -E-S-N-S(P)-V-D-T-S- | 44       |
| Phosphorylase          | No                                      | -K-E-I-S(P)-V-R-G-L- | 36       |
| Skeletal muscle light chain | No                                   | -E-G-S-S(P)-N-V-F-S- | 45       |
| Cardiac muscle light chain | No                                   | -E-G-S-S(P)-N-V-S- | 45       |

| Table II                |
|-------------------------|
| Comparison of phosphorylation site sequences |

| Protein                | Substrate for CaM-dependent synthase kinase | Sequence | Reference |
|------------------------|------------------------------------------|----------|-----------|
| Smooth muscle light chain | Yes                                     | -R-A-T-S(P)-N-V-F-A- | 43       |
| Synthase site 2        | Yes                                     | -R-T-L-S(P)-V-S-S-L- | 11       |
| Synthase site 1b       | Yes                                     | -E-S-N-S(P)-V-D-T-S- | 44       |
| Phosphorylase          | No                                      | -K-E-I-S(P)-V-R-G-L- | 36       |
| Skeletal muscle light chain | No                                   | -E-G-S-S(P)-N-V-F-S- | 45       |
| Cardiac muscle light chain | No                                   | -E-G-S-S(P)-N-V-S- | 45       |

| Table III               |
|-------------------------|
| Substrate specificities of CaM-dependent synthase kinase and myosin light chain kinase |

Phosphorylation reactions contained 50 mM Hepes, pH 7.5, 1 µM CaM, 0.5 mM CaCl2, 0.3 mM NaCl, 10 mM MgCl2, 0.5 mM [γ-32P]ATP (450 cpm/pmol), and either 1.7 µM glycogen synthase, 26 µM smooth muscle myosin light chain (20,000 daltons), or 11.8 µM smooth muscle, myosin plus the indicated kinase.

| Substrate                | CaM-dependent synthase kinase | Smooth muscle myosin light chain kinase |
|-------------------------|-------------------------------|----------------------------------------|
| Glycogen synthase       | 17.2                          | <0.1                                   |
| Smooth muscle light chain | 9.3                          | 4.8                                    |
| Smooth muscle myosin    | <0.1                          | 2.7                                    |
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Based upon a Stokes radius of about 70 Å, a sedimentation coefficient of 10.6 S, and an assumed partial specific volume of 0.725 cm³/g, a M, of about 300,000 and a frictional ratio of about 1.6 were calculated for calmodulin-dependent glycogen synthase kinase. An unequivocal subunit structure has not been defined; however, the major bands observed after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate corresponded to molecular weights of approximately 50,000 to 53,000. Presently, it has not been established whether these represent two different subunits or result from phosphorylation or proteolysis of a single subunit.

Liver calmodulin-dependent glycogen synthase kinase appears to be distinct from other reported kinases. This enzyme is active toward skeletal muscle glycogen synthase, liver glycogen synthase, and purified smooth muscle myosin light chain, but inactive toward skeletal muscle phosphorylase b, liver phosphorylase b, myosin light chain from skeletal and cardiac muscle, native smooth muscle myosin, liver pyruvate kinase, liver phosphofructokinase, histone IIA, casein, and the regulatory subunit of type II cAMP-dependent protein kinase (14, 15). The ability of this kinase to phosphorylate isolated light chains of smooth muscle but not intact myosin is similar to the cyclic AMP-dependent protein kinase (31) and indicates that the reaction is probably not of physiological significance. Both liver calmodulin-dependent glycogen synthase kinase and skeletal muscle phosphorylase kinase can phosphorylate the serine which is located 7 residues from the NH₂-terminus of skeletal muscle glycogen synthase (site 2) (11). In addition to site 2, the calmodulin-dependent glycogen synthase kinase also phosphorylated site 1b (Fig. 9). The decrease in the synthase activity ratio can probably be attributed to the phosphorylation of site 2. Only phosphorylase kinase can phosphorylate the serine 14 residues from the NH₂-terminus of phosphorylase b. There is considerable homology between residues 4-15 from the NH₂-terminus of glycogen synthase (11) and residues 11-22 in phosphorylase b (36). Therefore, it is interesting to note that the calmodulin-dependent glycogen synthase kinase will phosphorylate glycogen synthase but not the homologous sequence in phosphorylase b. An interesting common feature of all the known substrates of liver CaM-dependent synthase kinase is the sequence -R-X-X-S(P)- as shown in Table II. Studies using synthetic peptide substrates may provide an answer to these specificity differences.

It has recently been shown that the subunit structure of liver phosphorylase kinase is similar if not identical with the skeletal muscle enzyme including the presence of calmodulin (37). While phosphorylation of glycogen synthase by calmodulin-dependent glycogen synthase kinase is completely dependent on calmodulin and inhibited by the phenothiazine trifluoperazine, this is not the case for liver phosphorylase kinase. The two enzymes also differ in the effect of excess magnesium. Magnesium in concentrations exceeding the concentration of ATP profoundly inhibited both nonactivated and activated liver phosphorylase kinase (37, 38). In contrast, the calmodulin-dependent glycogen synthase kinase was most active with the concentration of magnesium greatly exceeding that of the ATP. Calmodulin-dependent glycogen synthase kinase is also distinguished from liver phosphorylase kinase by their respective observed pH optima for phosphorylation and the reported holoenzyme molecular weights (37). Furthermore, the calmodulin-dependent synthase kinase has been purified from the livers of the New Zealand strain of rat with a glycogen storage disease (gsd/gsd) characterized by the absence of liver phosphorylase kinase (39).

Calmodulin-dependent glycogen synthase kinase also differs from the studies detailed in this paper pertain to that fraction (unless otherwise stated). The calmodulin-sensitive synthase kinase recovered was eluted from the calmodulin-Sepharose column (see Fig. 4), about 20% of the total activity, was not due to overloading the column since it did not adsorb when reapplied to different affinity columns varying the concentration of calcium and the pH. This fraction has not yet been studied extensively. Based upon data obtained utilizing other calmodulin-dependent enzymes, some possible explanations for the resolution of two glycogen synthase kinases with differing affinities for calmodulin by chromatography on calmodulin-Sepharose are as follows: the existence of isoenzymes of the enzyme (33), the existence of phospho- and dephospho-forms of the enzyme (34), or the partial degradation by proteolysis of the native enzyme (35). Only fresh, unfrozen livers were used for the preparation of the calmodulin-dependent glycogen synthase kinase. In addition, the livers were homogenized in the cold in the presence of 0.25 M sucrose, 4 mM EGTA, 2 mM EDTA, and several protease inhibitors. Also, several protease inhibitors and either EDTA and/or EGTA were included in the buffers throughout the early purification stages. Consequently, although it cannot be conclusively ruled out, it is unlikely that the calmodulin-dependent activity which did not bind to the affinity column is a proteolytic breakdown product.

The relationship between the two calmodulin-dependent glycogen synthase kinases must await further studies on the structural and enzymatic characterization of the two forms. We previously reported (Fig. 1 of Ref. 14) that chromatography on Sepharose 6B resulted in the resolution of the calmodulin-dependent kinase from a calmodulin-independent synthase kinase. In the current purification scheme, the calmodulin-independent synthase kinase was removed by the 40 mM NaCl wash of the DEAE-Sepaharose column step. This glycogen synthase kinase, which is unaffected by either cAMP or calmodulin, has not been studied sufficiently to comment further.

DISCUSSION

Calmodulin-dependent glycogen synthase kinase was isolated from rabbit liver and purified 4500-fold to a specific activity of 2.23 μmol of 32P/mol of subunit using the calmodulin-dependent synthase kinase. The 32P-synthase was digested with trypsin (1 mg/ml for 5 h) and the peptides were mapped by reverse phase high performance liquid chromatography using a gradient of 1-propanol (0-25% in 100 min, 25-50% in 10 min) in 0.1% trifluoroacetic acid.

FIG. 9. Peptide mapping of phosphorylation sites. Glycogen synthase was phosphorylated to about 1 mol of 32P/mol of subunit using the calmodulin-dependent synthase kinase. The 32P-synthase kinase was digested with trypsin (1 mg/ml for 5 h) and the peptides were mapped by reverse phase high performance liquid chromatography using a gradient of 1-propanol (0-25% in 100 min, 25-50% in 10 min) in 0.1% trifluoroacetic acid.

5 M. F. Payne, unpublished data.
shown to be more potent in inactivating glycogen synthase than in activating phosphorylase.

In conclusion, a calcium, calmodulin-dependent protein kinase which phosphorylates and inactivates glycogen synthase has been purified from liver. This enzyme may mediate the actions of \( \alpha \)-adrenergic agonists, vasopressin, and angiotensin II on glycogen synthase in liver. In one possible model, these agents interact with specific receptors located on the plasma membrane; this results in the mobilization of calcium from mitochondria (Fig. 10). Calcium then presumably binds to the ubiquitous calcium-binding protein, calmodulin, forming an active conformer. This active conformer subsequently binds to the inactive calmodulin-dependent glycogen synthase kinase. As a result, an active enzyme would be formed which would then phosphorylate and inactivate glycogen synthase.

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Addendum—After this manuscript was submitted, an article on the purification and characterization of rabbit liver CaM-dependent synthase kinase was published (Ahmed, Z., DePaoli-Roach, A. A., and Roach, P. J. (1982) J. Biol. Chem. 257, 8348-8355). The results of that paper are in general agreement with our earlier observations (14, 15) and this present manuscript except for the protein substrate specificity of the kinase. Ahmed et al. find substantial phosphorylation of casein and phosvitin (68 and 34%, respectively, relative to synthase) whereas we find less than 10% relative activity toward these proteins (14).

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