Purification and Characterization of a Rabbit Liver Calmodulin-dependent Protein Kinase Able to Phosphorylate Glycogen Synthase*

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Zafeer Ahmad, Anna A. DePaoli-Roach‡, and Peter J. Roach

From the Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46223

A calmodulin-dependent protein kinase has been extensively purified from rabbit liver by the criterion of its ability to phosphorylate muscle glycogen synthase. The enzyme bound to phosphocellulose, DEAE-cellulose, and blue dextran-agarose. The enzyme also bound, in a Ca²⁺-dependent manner, to a calmodulin-agarose affinity column. Expression of activity required the presence of both calmodulin and Ca²⁺, with half-maximal activation occurring at approximately 80 nM calmodulin. Triethylammonium, 50 μM, completely inhibited the enzyme. Enzyme activity was associated with two polypeptide species of apparent molecular weights 53,000 and 51,000. The molecular weight of the native enzyme was approximately 500,000, as judged from gel filtration, and 275,000 as determined from sucrose density gradient sedimentation, suggesting an oligomeric structure. Incubation of the enzyme with ATP and Mg²⁺ led to phosphorylation of the constituent polypeptides and an accompanying decrease in electrophoretic mobilities in the presence of sodium dodecyl sulfate. The protein kinase had an apparent Kₘ for ATP of 27 μM and had half-maximal activity at 0.75 mM Mg²⁺. The protein kinase phosphorylated muscle glycogen synthase to a stoichiometry of greater than 1 phosphate/subunit with significant inactivation. Phosphatase was introduced into at least two sites on the glycogen synthase. Another effective substrate for the protein kinase was the smooth muscle 20,000-dalton myosin light chain. The enzyme had modest activity toward cardiac myosin light chains, histone, and either skeletal muscle or liver glycogen phosphorylase. The enzyme is, therefore, distinguishable from the two best characterized calmodulin-stimulated protein kinases, phosphorylase kinase and myosin light chain kinase, and represents a distinct category of such enzymes.

Multiple phosphorylation of the subunit of glycogen synthase (EC 2.4.1.11) occurs in vitro through the action of some five or more distinct protein kinases (see Refs. 1–4 for a review) and may afford a complex regulation of this rate-limiting enzyme of glycogen synthesis. Muscle enzymes have been most extensively studied, and three major classes of glycogen synthase kinase have been identified: 1) cyclic AMP-dependent protein kinase, 2) phosphorylase kinase, and 3) a cyclic nucleotide and Ca²⁺-independent category that includes PC₆₇ (6, 6), PC₄₄ (5, and the glycogen synthase kinase-3 (GSK-3) of Cohen and collaborators (7, 8). The enzymology of liver glycogen synthase and its protein kinases is much more poorly understood. Since the regulation of liver glycogen metabolism displays both important similarities to and differences from that of muscle, it is of considerable significance to understand to what extent potential regulatory elements such as protein kinases are analogous in the two tissues. Of the cyclic nucleotide and Ca²⁺-independent category, liver does contain PC₆₇ (9) probably an activity analogous to PC₄₄ and most likely an enzyme corresponding to GSK-3 (10). Both cyclic AMP-dependent protein kinase and phosphorylase kinase are known to occur in liver. It is not completely clear to what extent the liver phosphorylase kinase is a glycogen synthase kinase though one report does document such activity (11). Rabbit liver contains another Ca²⁺-dependent glycogen synthase kinase, first described by Payne and Soderling (12, 13), that is a calmodulin-requiring enzyme. No clearly analogous enzyme has so far been reported in muscle. Special interest in Ca²⁺-activated glycogen synthase kinases, especially from liver tissue, rests in the suggestion of Ca²⁺-mediated activation of glycogen synthase in isolated hepatocytes stimulated by α-adrenergic agonists, vasopressin or angiotensin (14–16). We have succeeded in confirming the existence of the liver calmodulin-dependent protein kinase and in purifying the enzyme close to apparent homogeneity. This report provides an initial characterization of the enzyme, in particular showing that the enzyme consists of two polypeptides of apparent molecular weights 51,000 and 53,000 arranged in an oligomeric structure.

EXPERIMENTAL PROCEDURES

Assays of Enzyme Activity—Glycogen synthase was assayed by the method of Thomas et al. (17). The percentage of 1 activity is the ratio of activity measured in the absence of glucose-6-P to that in the presence of 7.2 mM glucose-6-P, multiplied by 100.

Protein kinase assays were run by adding 10 μl of protein kinase solution to 40 μl of a solution, pH 7.5, containing 50 mM Tris-Cl, 1.25 mM EDTA, 0.5 mM EGTA, 7.5 mM Mg(CH₂COO)₂, 0.125 mM [γ-³²P] ATP (500–1,500 cpm/μmol), and protein substrate. Glycogen synthase (for the standard assay) was present at a final concentration of 0.2 mg/ml. Protein kinase activity was terminated by adding 50 μM TLCK, W-p-tosyl-L-lysine chloromethyl ketone HCI.

The term "glycogen synthase kinase" is used generically to describe any enzyme capable of phosphorylating glycogen synthase in vitro. The designations PC₆₇ and PC₄₄ are operational names of protein kinases as explained in Ref. 5.

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The abbreviations used are: EGTA, ethylene glycol bis(β-amino-ethel ether)N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; TLCK, N-p-tosyl-1-lysine chloromethyl ketone HCI.
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mg/ml, and other substrates were as indicated. When included, calmodulin and CaCl₂ were present at final concentrations of 0.57 μM and 0.4 mM (in excess of EGTA), respectively. Because of the very low yield of protein in the purified enzyme, determination of exact concentration was difficult. We estimate, however, that the calmodulin-dependent protein kinase was normally in the range of 0.05–0.5 μg/ml. Incubation was for 20 min at 30°C. Variations from the above protocol of 20% (w/v) trichloroacetic acid containing 1 mM ATP and 2 mM potassium pyrophosphate. The filter paper squares were washed first for 15 min in 10% (w/v) trichloroacetic acid and then three times for 20 min each in 5% (w/v) trichloroacetic acid. After a final wash, 5 min, in diethyl ether, the papers were dried, placed in a scintillator of 0.5% (w/v) diphenylxazole in toluene, and counted in a Beckman LS7000 scintillation counter. Protein kinase activity expressed as picomoles/assay is referred to as total activity (see text and Methods).

Proteins—Glycogen synthase was purified from rabbit skeletal muscle as described previously (19). Phosphorylase from the same source was purified by a modification of the method of Fischer and Kress (20). The final preparation contained a protein kinase activity of much lower specific activity (Fig. 2). In fact, in earlier preparations, the enzyme was eluted with buffer A plus 7 mM EGTA (Fig. 2). Further elution of the column with buffer A plus 7 mM EGTA and 0.2 M NaCl yielded greater purity at this stage. The smaller second peak of calmodulin-stimulated activity from the gel filtration step was applied in 6.2 ml fractions of 1 ml to a column (4 ml) of calmodulin-agarose. After washing with 60 ml of buffer A, elution was initiated with buffer A plus 7 mM EGTA and 0.2 M NaCl. The flow rate was 5 ml/h, and 1 ml fractions were collected. Glycogen synthase kinase activity in the presence of Ca²⁺ and calmodulin, was determined (assay 2) and is shown by the solid line. The A₂₅₀ is indicated by the dashed line. The excluded volume fell between fractions 37 and 38.

**FIG. 1.** Gel filtration. The partially purified calmodulin-stimulated glycogen synthase kinase was applied, in 6.2 ml fractions of 1 ml to a column (2.6 x 82 cm) of Bio-Gel A-1.5m and eluted at a flow rate of 24 ml/h. Fractions of 4.5 ml were collected and assayed for glycogen synthase kinase activity either in the absence (○) or presence (●) of Ca²⁺ and calmodulin (assay 2). The A₂₅₀ is indicated by the dashed line. The excluded volume fell between fractions 37 and 38.

**FIG. 2.** Calmodulin-agarose chromatography. Pooled calmodulin-stimulated activity from the gel filtration step was applied in the presence of 6 mM CaCl₂ to a column (4 ml) of calmodulin-agarose. The first arrow indicates the start of elution with buffer A plus 7 mM EGTA, and the second arrow indicates elution with buffer A plus 7 mM EGTA and 0.2 M NaCl. The flow rate was 5 ml/h, and fractions of 1 ml were collected. Glycogen synthase kinase activity in the presence of Ca²⁺ and calmodulin was determined (assay 2) and is shown by the solid line. The A₂₅₀ is indicated by the dashed line.

**FIG. 3.** Blue dextran-agarose chromatography. The peak of activity from the calmodulin-agarose column was pooled and applied to a column (4 ml) of blue dextran-agarose equilibrated with buffer A. After washing with 70 ml of buffer A, elution was initiated with buffer A plus 0.3 M NaCl (starting at fraction 1). The flow rate was 5 ml/h, and fractions of 1 ml were collected. Glycogen synthase kinase activity, in the presence of Ca²⁺ and calmodulin, was determined using assay 2 and is shown by the solid line. The A₂₅₀ is indicated by the dashed line.

In our initial work, we utilized KCl for the various chromatographic elutions but have switched to NaCl because of the lower solubility of potassium dodecyl sulfate, a problem for polyacrylamide gel electrophoretic analysis in the presence of SDS.

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*Note:* The above text is a segment from a scientific paper discussing the purification and characterization of calmodulin-stimulated glycogen synthase kinase. The details include methods of purification, the role of calmodulin, and different analytical techniques used to assess the activity and purity of the enzyme. The text is interspersed with figures and diagrams illustrating the purification steps and activity assays. The objective is to provide a comprehensive understanding of the purification process and the properties of the enzyme.
A purification table is shown in Table I. We must note that determination of protein at the latter stages of the purification was very difficult because of the low amounts and concentrations of protein involved, and our values are not of the highest accuracy. Specific activities of the purified enzyme were estimated to be in the range of 4 to 5 nmol/min/mg. Attempts to concentrate the dilute fractions of the purified enzyme have so far led to significant losses of activity. Storage at -70 °C or, with 50% (v/v) glycerol at -20 °C, has been applied with some success although loss of activity with storage has been observed.

We note that earlier purification schemes had employed DEAE-cellulose chromatography with which the enzyme eluted at 0.12 M KCl. The blue dextran-aragose chromatography has been substituted for this step.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis in the presence of SDS followed the method of Laemmli (24) with 0.75-mm thick slab gels and the indicated percentage of acrylamide. Gels were stained to visualize protein by the ultraviolet absorbing method (25). Molecular weight standards were phosphorylase, bovine serum albumin, ovalbumin, and trypsinogen which were assigned subunit molecular weights of 97,000, 68,000, 45,000, and 24,000, respectively. Where necessary, autoradiograms were made by placing dried gels in contact with Cronex 4 x-ray film in Quanta III intensifying cassettes (DuPont) at -70 °C. Scanning of either dried gels or autoradiograms was with a Beckman DU-8 spectrophotometer with a white light source.

**Autophosphorylation of Calmodulin-dependent Protein Kinase**—The protein kinase was incubated in a reaction mixture containing 15 mM 1,4-piperazinediethanesulfonic acid, 0.75 mM EGTA, and 30 mM Tris, pH 7.2, and, where indicated, 0.11 mM [γ-32P]ATP (906 cpm/pmol) and 6 mM MgCl2. When present, CaCl2 and calmodulin were at 1.1 mM and 10 μg/ml, respectively. At the desired times, aliquots of reaction mixture were removed and mixed with electrophoresis sample buffer to give a final concentration of 1% (w/v) SDS and heated for 4 min at 100 °C. These samples were later analyzed by polyacrylamide gel electrophoresis in the presence of SDS (see Fig. 7).

**Other Materials and Methods**—Protein determination for the calmodulin-dependent protein kinase was achieved with Coomasie-staining assay (26). CNBr fragmentation and partial tryptic degradation of glycogen synthase was as described previously (5, 6). The concentration of [γ-32P]ATP was routinely determined by UV absorbance. Calmodulin-agarose was prepared as reported previously (27). [γ-32P]ATP was obtained from Amersham Corp., and trifluoperazine was from Smith, Kline and French.

**RESULTS**

**Purification of Calmodulin-dependent Protein Kinase**—The methods detailed under "Experimental Procedures" have allowed the extensive purification of the protein kinase, close to apparent homogeneity. The exact degree of purification is difficult to estimate because of the multiplicity of glycogen synthase kinases present at the early stages. In addition, calmodulin-stimulated activity was not readily detectable until partial purification had been achieved. Some degree of stimulation was usually observed in the 100,000 X g supernatant but only after phosphocellulose chromatography was a significant and reproducible stimulation found. Referring purification to the calmodulin-dependent component of the phosphocellulose eluate gives a 500-fold further purification with 15% yield (Table I). If instead purification is referred simply to glycogen synthase kinase specific activity (in the presence of Ca2+ and calmodulin) of the crude extract, 20,000-fold purification with 1.1% yield is obtained. The true values probably lie somewhere between these extremes.

The phosphocellulose eluate, only partially calmodulin-dependent, was resolved into two fractions of glycogen synthase kinase activity by the subsequent gel filtration step (Fig. 1). One of these fractions was totally dependent on Ca2+ and calmodulin for activity and retained this property through further purification steps. The second protein kinase fraction was unaffected by Ca2+ and calmodulin. Work continues on this calmodulin-independent enzyme, but initial evidence suggests that it is a distinct protein kinase. The purification scheme of Table I has evolved from earlier trials, differing in two main ways. First, we initially eluted the enzyme from the calmodulin-agarose affinity column with an excess of EGTA in the presence of 0.2 M KCl. It was found though that the enzyme could be more specifically eluted with EGTA alone (Fig. 2). Secondly, our first publications used DEAE-cellulose chromatography, whereas later the blue dextran agarose step. It should be noted that the purification of the calmodulin-dependent protein kinase diverges from that of a set of liver Ca2+- and cyclic nucleotide independent glycogen synthase kinases ("PC" series enzymes; see Refs. 5, 6, and 7) at the stage of the pH 6.0 precipitation, the calmodulin-stimulated enzyme being found in the precipitate and the other protein kinases in the supernatant.

**Molecular Weight and Polypeptide Composition**—From calibration of the gel filtration column (Fig. 1) used in the purification (with thyroglobulin, aldolase, bovine serum albumin, and ovalbumin) an apparent molecular weight of approximately 500,000 was obtained. Sucrose density gradient sedimentation of the purified enzyme gave an apparent molecular weight of 275,000 (Fig. 4).

Analysis, by polyacrylamide gel electrophoresis in the presence of SDS, of enzyme eluted from the calmodulin-agarose column (Fig. 2) or the blue dextran-agarose column (Fig. 3) indicated the presence of two major polypeptide species visualized with the silver staining technique (Fig. 5). These polypeptides had apparent molecular weights of 51,000 and 53,000 and will be designated α and β, respectively. Other polypeptides are scarcely visible in Fig. 5 except for low molecular weight species running close to the tracking dye. This material has been variable in amount and was partially removed by the blue dextran column. The α and β polypeptides, in contrast, correlated strictly with enzyme activity in several chromatographic separations, besides those shown, and in several different enzyme purifications. Furthermore,

### Table I

| Purification of calmodulin-stimulated glycogen synthase kinase | Total activity | Protein | Specific activity | Yield | Purification | Purification |
|---------------------------------------------------------------|---------------|---------|------------------|-------|--------------|--------------|
|                                                              | -Ca2+, -calmodulin | Protein | -Ca2+, + calmodulin | mg | nmol/min/mg | % | fold | fold |
| Crude extract                                                | 564           | 851     | 20,800           | 0.04 | 1            | 1            |
| pH 6.0 precipitate                                            | 274           | 275     | 5,950            | 0.046 | 1.2          | 2.3          |
| 100,000 X g supernatant                                       | 189           | 173     | 1,827            | 0.09 | 2.3          | 29.1         |
| Phosphocellulose eluate                                       | 30            | 58      | 55               | 1.5  | 100          | 1            |
| Bio-Gel A-1.5m eluate                                         | 0             | 53      | 12.5             | 4.3  | 92           | 3            |
| Calmodulin-agarose eluate                                    | 0             | 18      | 0.083            | 217  | 31           | 145          |
| Blue dextran-agarose eluate                                   | 9             | 9.6     | 0.012            | 797  | 16           | 531          |

*Calculated on the basis of the Ca2+ - and calmodulin-dependent component of the activity, starting at the phosphocellulose eluate.
2 Calculated on the basis of glycogen synthase kinase activity in the presence of Ca2+ and calmodulin, starting at the crude extract.
3 The main fraction, eluting with buffer A + 7 mM EGTA (see Fig. 2).
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The upper band (β-polypeptide) is in fact slightly thicker. Intense but, upon closer scrutiny, this may be offset by the fact that a portion of the purification peptides are for estimating stoichiometry. Nonetheless, the technique gave a ratio of intensities between enzyme activity and the dient sedimentation (Fig. 4) showed a similar correspondence to 1:1.04 to 1:1.13. It should be pointed out that it is not clear how valid such determinations with silver stained polyacrylamide gels are.

**Fig. 4.** Sucrose density gradient sedimentation. Purified calmodulin-dependent protein kinase, 100 μl containing approximately 1 μg of protein, was applied on a 5 to 20% sucrose gradient in buffer A plus 0.2 M KCl without glycerol. After centrifugation for 5.5 h at 48,000 rpm in a Beckman SW 20.1 rotor, 27 fractions were collected from the bottom of the tube (0.1 ml/fraction), and an aliquot (10 μl) was assayed for glycogen synthase activity in the presence of Ca²⁺ and calmodulin using assay 2. Fraction 1 corresponds to the bottom of the gradient. Standards were run on parallel gradients and were located (as indicated by the arrows) by UV absorption. The standards were (i) phosphorylase kinase; (ii) thyroglobulin; (iii) phosphorylase; (iv) aldolase; (v) lactate dehydrogenase; (vi) ovalbumin.

**Fig. 5.** Gel electrophoretic analysis of calmodulin-dependent protein kinase. Fractions recovered from the calmodulin-agarose chromatography (Fig. 2) and blue dextran-agarose chromatography (Fig. 3) were analyzed by polyacrylamide gel electrophoresis in the presence of SDS and stained by the silver technique. A shows the analysis (9% acrylamide) of the equivalent of 15 μl of fractions 7 to 13 (tracks 1 to 7, respectively) from the elution shown in Fig. 2. B shows the analogous analysis (but with 10% acrylamide) of the equivalent of 15 μl of fractions 6 to 12 (tracks 1 to 7, respectively) from the elution shown in Fig. 3.

**Fig. 6.** Gel electrophoretic analysis of fractions from sucrose density gradient sedimentation. The equivalent of 50 μl of fractions 13 to 19 (tracks 1 to 7, respectively) from Fig. 4 was analyzed by polyacrylamide gel electrophoresis (10% polyacrylamide) in the presence of SDS and visualized by the silver stain.

**Fig. 7.** Autophosphorylation of calmodulin-dependent protein kinase. The protein kinase (approximately 15 μg/ml) was incubated: (i) with no additions (tracks 1 and 4); (ii) in the presence of [γ-32P]ATP and Mg²⁺ (tracks 2 and 5); or (iii) in the presence of [γ-32P]ATP and Mg²⁺, and Ca²⁺ and calmodulin (tracks 3 and 6). Further details are given under “Experimental Procedures.” The equivalent of 15 μl of reaction mixture was analyzed by polyacrylamide gel electrophoresis in the presence of SDS (15% acrylamide) after 30 s (tracks 1 to 3) and 10 min (tracks 4 to 6) of incubation. The gel was stained by the silver technique (B), and an autoradiogram was made (A) of the dried gel. The lower dark line in B corresponds to the migration of the tracking dye, and the bars at the right-hand side indicate apparent molecular weights of 54,500 and 53,000.

Electrophoretic analysis of fractions from sucrose density gradient sedimentation (Fig. 4) showed a similar correspondence between enzyme activity and the α and β polypeptides (Fig. 6). Densitometric scanning of gels stained with the silver technique gave a ratio of intensities α/β ranging from 1.08 to 1.12 or, normalizing for apparent molecular weights, molar ratios α/β of 1.94 to 1.13. It should be pointed out that it is not clear how valid such determinations with silver stained polypeptides are for estimating stoichiometry. Nonetheless, the α/β ratio so determined was constant through the last stages of the purification (e.g., Fig. 5) and between different preparations. We suggest that enzyme activity is associated with the α and β polypeptides.

**Autophosphorylation**—When the protein kinase was incubated with [γ-32P]ATP and Mg²⁺, in the presence of Ca²⁺ and calmodulin, phosphorylation of constituent polypeptides occurred (Fig. 7). From gel electrophoretic analysis, 32P was first observed with mobility close to that of the β-polypeptide, i.e., apparent Mₚ = 53,000) and later in a more slowly migrating species (apparent Mₚ = 54,500). Radioactivity also appeared, somewhat more slowly, close to the dye front (see Fig. 7) and represented the phosphorylation of the low molecular weight contaminating material discussed above. Indeed, the extent of this latter phosphorylation varied for different preparations. By cutting gels, such as shown in Fig. 7, in the regions corresponding to the molecular weight range 53,000-54,500, and counting the associated radioactivity, it was estimated that up to approximately four phosphates per original α plus
were incorporated (data not shown), a rather high level of phosphorylation.

Concomitant with the phosphorylation reaction(s) was a significant alteration in the pattern of the silver staining material on the gel (Fig. 7). Already after 30 s, no polypeptide was seen with the mobility of the α-polypeptide and at longer times, the two main species visible had apparent molecular weights of 53,000 and 54,500, in correspondence with the 32P-labeling. As shown in Fig. 7, no phosphorylation or alterations in electrophoretic mobilities occurred either when Ca2⁺ and calmodulin were omitted or when the enzyme was incubated without Ca2⁺, calmodulin, ATP, and Mg2⁺. In addition, incubation with Ca2⁺ and calmodulin, but without ATP and Mg2⁺, had no effect on electrophoretic mobilities (not shown). Using the standard assay, no significant change in activity accompanied the phosphorylation reaction (not shown). The changes in electrophoretic behavior correlated with the incorporation of 32P, but the exact sequence of events has yet to be worked out. For instance, we cannot distinguish whether only α or both α and β acquire altered mobility upon phosphorylation.

The reduction of the electrophoretic mobility of a polypeptide by phosphorylation may not be a general occurrence but neither is it a novel observation. A prominent example is the regulatory subunit of type II cyclic AMP-dependent protein kinase (28, 29). The same phenomenon was recently described for the GSK-3 enzyme of Hemmings et al. (8) which also self-incorporated as many as four phosphates per subunit.

Activation by Calmodulin—The enzyme was totally dependent on the presence of Ca2⁺ and calmodulin for activity also toward exogenous substrates and was completely inhibited by 50 μM trifluoperazine (Table II) which is known to inhibit calmodulin-sensitive enzymes (30). The dependence of enzyme activity on calmodulin concentration (Fig. 8) indicated half-maximal stimulation at approximately 80 nM. Interaction with calmodulin was further indicated by the fact that, during the purification, the enzyme bound to a calmodulin-agarose affinity column in the presence of Ca2⁺ and was eluted with an excess of EGTA (Fig. 2).

Substrate Specificity—The activity of the protein kinase toward a number of protein substrates is shown in Table II. The most significant substrate, other than skeletal muscle glycogen synthase, was the smooth muscle 20,000-dalton light chain. Glycogen synthase and myosin light chain kinase activities were also seen to co-elute from a DEAE-cellulose column and also from the calmodulin-agarose column (not shown). The calmodulin-stimulated glycogen synthase kinase was not, however, effective in phosphorylating cardiac myosin light chains (Table II). This behavior contrasts with legitimate smooth muscle myosin light chain kinase which did not phosphorylate glycogen synthase but did phosphorylate cardiac light chain (Table II). The glycogen synthase kinase also phosphorylated, at moderate initial rates, phosvitin and casein in a Ca²⁺- and calmodulin-dependent reaction (Table II).

No phosphorylation of histone, either in the presence or absence of 10 μM cyclic AMP, was observed with the protein kinase. Heparin (0.25 μg/ml), a potent inhibitor of certain protein kinases (31, 32), had no effect on this calmodulin-dependent enzyme. An important negative result was the inability of the protein kinase to phosphorylate rabbit liver or skeletal muscle phosphorylase, under conditions where both substrates could be phosphorylated by muscle phosphorylase.

![Fig. 8. Dependence of protein kinase activity on calmodulin. Glycogen synthase kinase activity was measured (assay 2) in the presence of 0.4 mM Ca⁺⁺ and the indicated concentration of calmodulin.](image)

![Fig. 9. Dependence of protein kinase activity on ATP and Mg²⁺ concentrations. Glycogen synthase kinase activity was measured by the standard methods (assay 2) except that either the ATP concentration (left panel) or the Mg²⁺ concentration (right panel) were varied as indicated. Note that total Mg²⁺ is plotted on the abscissa.](image)

| Table II |
|---|
| **Substrate** | **Calmodulin-stimulated protein kinase** | **Myosin light chain kinase** |
| (mg/ml) | pmol/assay | pmol/assay |
| Glycogen synthase (0.2) | 106 | 0 |
| Glycogen synthase (0.2) plus 50 μM trifluoperazine | 0 | — |
| Muscle phosphorylase (2) | 5.9 | — |
| Phosvitin (2) | 34.5 | — |
| Casein (2) | 58.3 | — |
| Histone (cyclic AMP) (3) | 0 | — |
| Smooth muscle myosin light chain (0.2) | 108 | 140 |
| Cardiac muscle light chains (0.1) | 0 | 34 |

" The substrate concentration (mg/ml) is indicated in parentheses.

" Both protein kinases had undetectable activity toward all substrates in the absence of Ca²⁺ and calmodulin.

" Not determined.

![Table III](image)

| Table III |
|---|
| **Activity of calmodulin-stimulated protein kinase with phosphorylase** |
| Protein kinase | Protein substrate | Activity |
| | | mg/ml | pmol/assay |
| Liver calmodulin-stimulated protein kinase | Muscle glycogen synthase (0.2) | 41 |
| | Liver phosphorylase (0.4) | 0 |
| Muscle phosphorylase kinase | Muscle phosphorylase (2) | 146 |
| | Liver phosphorylase (0.4) | 6 |

![Fig. 8. Dependence of protein kinase activity on calmodulin. Glycogen synthase kinase activity was measured (assay 2) in the presence of 0.4 mM Ca⁺⁺ and the indicated concentration of calmodulin.](image)

![Fig. 9. Dependence of protein kinase activity on ATP and Mg²⁺ concentrations. Glycogen synthase kinase activity was measured by the standard methods (assay 2) except that either the ATP concentration (left panel) or the Mg²⁺ concentration (right panel) were varied as indicated. Note that total Mg²⁺ is plotted on the abscissa.](image)
kinase (Table III).

**Nucleotides and Mg**<sup>2+</sup>—The dependence of protein kinase activity on ATP concentration (Fig. 9) was hyperbolic and was characterized by an apparent $K_m$ of 27 μM. With [γ-<sup>32</sup>P]ATP at 25 μM, 200 μM GTP (unlabeled) had no effect on activity and 1 mM GTP caused 33% inhibition. These results suggest that GTP is unlikely to be an effective substrate. Variation of the Mg<sup>2+</sup> concentration indicated that approximately 0.75 mM was necessary for half-maximal activity (Fig. 9).

**Phosphorylation of Glycogen Synthase**—The calmodulin-dependent protein kinase phosphorylated glycogen synthase to a stoichiometry of greater than 1 phosphate/subunit (Figs. 10 and 11). The phosphorylation inactivated glycogen synthase. In Fig. 10, where a maximum level of approximately 1 phosphate/subunit was achieved, the percentage of I activity was decreased from 95% I to 45% I activity. From analysis of CNBr fragments of phosphorylated glycogen synthase, phosphate was shown to be introduced simultaneously into two 12,000-dalton fragments that have been described previously (5). Partial proteolysis with trypsin (as in Ref. 6) resulted in the formation of two 21,000-dalton fragments (Fig. 11) corresponding to the 12,000-dalton light chain, phospho-vitin and casein, is indicative of a somewhat broader substrate specificity for the protein kinase.

**DISCUSSION**

The present paper documents procedures for the extensive purification of a calmodulin-stimulated protein kinase from rabbit liver and some initial characterization of that enzyme (summarized in Table IV). The results confirm and extend the work of Payne and Soderling (12, 13) which, at the time of writing, appears to be the only study of this protein kinase. Our data largely concur with those of the earlier reports (12, 13), suggesting that the same enzyme was studied. One difference is that, in our studies, the protein kinase did not display the extremely restricted substrate specificity reported previously. Even though probably not of physiological significance, the calmodulin-dependent phosphorylation of smooth muscle 20,000-dalton light chain, phosphorylation (liver and muscle) is indicative of a somewhat broader substrate specificity for the protein kinase.

The results presented provide a first insight into the subunit structure of the enzyme and indicate that activity is associated with two polypeptides of apparent molecular weights 51,000 and 53,000. Their relative abundance is probably not far from equimolar although our analyses, based on densitometric scanning of stained polyacrylamide gels, cannot be considered definitive. We can say that the relative proportions of the polypeptides did not vary in different preparations. Such stoichiometric information would argue that the two polypeptides are distinct subunits of the enzyme. We hesitate from fully endorsing this hypothesis, however, because we know that the electrophoretic mobility of one or of both of the polypeptides is diminished upon phosphorylation. Could α and β be different phosphorylation states of the same poly-

![Fig. 10. Phosphorylation and inactivation of glycogen synthase.](image)

**Fig. 10. Phosphorylation and inactivation of glycogen synthase.** Glycogen synthase (0.2 mg/ml) was phosphorylated using standard reaction conditions in the presence of Ca<sup>2+</sup> and calmodulin. At the indicated times, aliquots were withdrawn (a) for determination of protein phosphorylation (assay 1) (●) and (b) for measurement of glycogen synthase percentage of I activity (□) after dilution 1:40 in 50 mM Tris·Cl, 5 mM EDTA, 2 mM EGTA, 60 mM β-mercaptoethanol, and 1 mg/ml of glycogen, pH 7.8.

![Fig. 11. Analysis of glycogen synthase phosphorylation of CNBr fragmentation.](image)

**Fig. 11. Analysis of glycogen synthase phosphorylation of CNBr fragmentation.** In an incubation similar to that of Fig. 5, aliquots of glycogen synthase were removed to determine total phosphorylation (assay 1; ●) or for fragmentation with CNBr, as described under “Experimental Procedures.” CNBr fragments were separated by gel electrophoresis with 18% acrylamide. Autoradiograms were scanned with a Beckman DU-8 spectrophotometer to quantitate the relative amounts of radioactivity in the 21,000-dalton fragment (△) and the 12,000-dalton fragment (○) of the glycogen synthase. The phosphorylation, in moles of <sup>32</sup>P/nmol of fragment, was then calculated and is plotted in the figure. A control reaction without added protein kinase was analyzed for total phosphorylation (○).

**TABLE IV**

| Summary of properties of the liver calmodulin-dependent protein kinase |
| --- |
| Specific activity<sup>a</sup> | 100–900 nmol/min/mg |
| Constituent polypeptides | α: 51,000 |
| (M<sub>a</sub>) | β: 53,000 |
| Stoichiometry<sup>b</sup> | α:β 1:1.03–1.13 |
| Native molecular weight | 500,000 |
| Gel filtration | 275,000 |
| Sucrose gradient centrifugation | 275,000 |
| Protein substrates<sup>c</sup> | Glycogen synthase (muscle) |
| Major | 20,000-dalton myosin light chain (smooth muscle) |
| Minor | Phosvitin, casein |
| Not phosphorylated | Histone, cardiac light chains, phosphorylase (liver and muscle) |
| Kinetic properties |  |
| Apparent $K_m$ (ATP) | 27 μM |
| $M_0.5$ (Mg<sup>2+</sup>) | 0.75 mM |
| $M_0.5$ (calmodulin) | 80 nM |
| Inhibition by Trifluoperazine (50 μM) | Yes |
| Heparin (0.25 μg/ml) | No |

<sup>a</sup> With standard assay using muscle glycogen synthase.
<sup>b</sup> Based on densitometric scanning of silver stained gels (see also text).
<sup>c</sup> Of course, this is based simply on those proteins so far tested.
<sup>d</sup> $M_0.5$, concentration required for half-maximal activity.
dependent protein kinase is able to phosphorylate liver glycogen as well as to ascertain specifically whether the calmodulin-dependent kinases that we have studied. Indeed, a variety of properties similarity in properties almost certainly exists between liver system of Merlevede's group retic mobility of the subunit, a property very reminiscent of not noted that GSK-3 was a monomer with subunit molecular weight 51,000 as judged by gel electrophoresis. However, reflect a deeper similarity between the two protein kinases is glycogen synthase kinases (reviewed in Refs. 1-4), the most calmodulin. Nonetheless, there is one intriguing property that will be tested.

The information on polypeptide composition rein-

glycogen phosphorylase, appears distinct from phosphorylase kinase. The enzyme that appears identical with the protein-activating chain kinase, by its ability to phosphorylate cardiac light chains and by its inability to phosphorylate glycogen synthase, is clearly distinguishable from the enzyme of this report. Likewise, the liver calmodulin-dependent glycogen synthase kinase, from its inability to phosphorylate muscle or liver glycogen phosphorylase, appears distinct from phosphorylase kinase. The information on polypeptide composition reinforces the above conclusions. We would suggest, then, that the enzyme described here is a representative of a third class of calmodulin-stimulated protein kinase that is approaching definition at the molecular level.

Phosphorylation of rabbit muscle glycogen synthase by the calmodulin-dependent kinase involves the introduction of phosphate, at roughly equivalent rates, into both an NH2-terminal 12,000-dalton CNBr fragment (containing serine-7, the site of action of phosphorylase kinase (11, 39, 40) and PCo4, (1, 5)) and a COOH-terminal 21,000-dalton CNBr fragment (containing the primary sites of action of cyclic AMP-dependent protein kinase (1, 5) and PCo4 (1, 6)). At least two sites on glycogen synthase are, therefore, phosphorylated. Such site specificity is different from that of other glycogen synthase kinases that we have studied. Indeed, a variety of properties distinguishes the enzyme of this report from other known glycogen synthase kinases (reviewed in Refs. 1-4), the most prominent characteristic being its stimulation by Ca2+ and calmodulin. Nonetheless, there is one intriguing property shared with the GSK-3 enzyme of Hemmings et al. (8), an enzyme that appears identical with the protein-activating factor, P0, of the ATP-Mg2+-dependent protein phosphatase system of Merlevede's group (41, 42). Hemmings et al. (8) noted that GSK-3 was a monomer with subunit molecular weight 61,000 as judged by gel electrophoresis. However, GSK-3 underwent autophosphorylation to at least four phosphates/subunit with an accompanying decrease in electrophoretic mobility of the subunit, a property very reminiscent of the enzyme of this report. Whether these common properties reflect a deeper similarity between the two protein kinases is an interesting possibility that will be tested.

While progress is being made on the molecular definition of the calmodulin-dependent glycogen synthase kinase, its physiologic role has yet to be established. Of course, the possibility that the enzyme could be an element in Ca2+-mediated controls of glycogen synthase activity in liver has been an important motivation for this and the earlier study of the enzyme. The criticism may be raised, however, that muscle glycogen synthase has been used as a substrate in all the work so far, as actually has been the case in most studies of nonmuscle glycogen synthase kinases. Though a degree of similarity in properties almost certainly exists between liver and muscle glycogen synthases, one cannot at present predict how far the two enzymes compare, in detail, as protein kinase substrates. Work is under way to address this broader question as well as to ascertain specifically whether the calmodulin-dependent protein kinase is able to phosphorylate liver glycogen synthase. Such study may help assess the possible role of the enzyme in the regulation of glycogen metabolism. For now, the present report provides an important first view of some structural features that will aid comparison of this liver calmodulin-dependent enzyme both with other calmodulin-dependent protein kinases and with other glycogen synthase kinases.

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