Purification of Rat Liver Phosphorylase Kinase*

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A rapid method for the purification of rat liver phosphorylase kinase 30,000-fold over homologous values is described. The method allows the isolation of a near homogeneous preparation of phosphorylase kinase initially associated with the glycogen pellet to be accomplished within 24 h. The enzyme has $M_\text{r} = 140,000$ and $M_\text{r} = 116,000$, and is closely paralleled by activation of the enzyme. The enzyme is phosphorylated by cAMP-dependent protein kinase: phosphate is incorporated into two of the subunits ($M_\text{r} = 149,000$ and $M_\text{r} = 118,000$) and is catalyzed by activation of the enzyme. The enzyme is partially inhibited by ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid and is stimulated by $10^{-4}$ to $10^{-6}$ M Ca$^{2+}$. The pH optimum of the nonactivated enzyme is 7.0. Activation by cAMP-dependent protein kinase does not appear to alter the Ca$^{2+}$ sensitivity of the enzyme. However, it results in a large increase in activity at pH 7 through 8, but not at pH below 6.5. Purified rat liver phosphorylase kinase thus shows many similarities to partially purified skeletal muscle phosphorylase kinase, but differs in respect to its incomplete inhibition by ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid and to the effects of phosphorylation by cAMP-dependent protein kinase on its pH activity profile and Ca$^{2+}$ sensitivity.

The study of liver phosphorylase kinase in crude liver extracts and in partially purified fractions has yielded inconsistent findings as to its molecular weight, susceptibility to activation by phosphorylation, and sensitivity to Ca$^{2+}$ (1). Not until recently has it been possible to partially purify a form of the enzyme having a molecular weight and regulatory properties similar to the well characterized rabbit skeletal muscle enzyme (2, 3). Although many similarities have been noted between the two enzymes, there also appear to be some distinct differences, i.e. pH optimum (2) and sensitivity to Ca$^{2+}$ (1). Whether these differences are real or are due simply to the study of impure preparations of the liver enzyme has been difficult to determine since pure preparations of the liver enzyme have not been available. We report here a rapid and relatively simple method of purification of rat liver phosphorylase kinase and some of the properties of the purified enzyme.

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MATERIALS AND METHODS

Heparin-Sepharose Cl-4B was from Pharmacia. The catalytic subunit of the beef heart cAMP-dependent protein kinase (4) was a gift from Dr. J. Corbin (Vanderbilt University, Nashville, TN) and the inhibitor of the cAMP-dependent protein kinase was purified according to the method of Ashby and Walsh (5). Rabbit skeletal muscle glycogen synthase I was a gift from Dr. T. Soderling (Vanderbilt University, Nashville, TN) and rat liver phosphorylase b was a gift from Dr. R. Uhing (Vanderbilt University, Nashville, TN). Rabbit skeletal muscle phosphorylase b was a gift from Dr. R. Uhing (Vanderbilt University, Nashville, TN). Polyethylene glycol 8000 (formerly "PEG 6000") was from Fisher. Rabbit skeletal muscle phosphorylase kinase was prepared by the method of Brostrom et al. (6) and further purified by DEAE-cellulose chromatography (7). Panheparin 1000 (1000 USP units of heparin/ml) was obtained from Abbott Laboratories. Histone Type II-A, histone Type V-S, phosphitin, and partially dephosphorylated casein were from Sigma. All other materials were from the sources described previously (8).

Phosphorylase Kinase Activity—The activity of phosphorylase kinase at 30°C was determined by measuring the transfer of $^3$P from [γ-$^3$P]ATP to skeletal muscle phosphorylase b. The standard 50-μl reaction mixture contained 20 mM triethanolamine HCl (pH 7.2) or 20 mM MES (pH 6.8) as indicated in the figures, 1 mM Mg(CH$_2$O)$_4$, 1 mM ATP, 2 x 10$^6$ cpm of [γ-$^3$P]ATP, 100 μg of skeletal muscle phosphorylase b, and 20–400 ng of phosphorylase kinase. The reaction was initiated with Mg(CH$_2$O)$_4$-ATP-[γ-$^3$P]ATP and terminated by transferring a 40-μl aliquot onto Whatman ET31 filter paper and further processed as in Ref. 3. Other reagents and conditions are given in the tables and figures.

Polyacrylamide Gel Electrophoresis—Electrophoresis in 7% polyacrylamide tube or vertical slab gels in the presence of 0.1% SDS was performed at pH 8.5 (8). The samples were prepared for electrophoresis by precipitation of the protein with cold 10% trichloroacetic acid and removing the liquid phase by aspiration. The precipitated protein was dissolved in 50 μl of sample buffer containing 2% SDS, 5% acrylamide, 2% 2-mercaptoethanol, 0.017% bromphenol blue, and Tris-glycine (0.025 M, 0.19 M, pH 8.5) and heated at 100°C for 5 min. For electrophoresis in tube gels, the samples were placed directly onto the 6% polyacrylamide gels and electrophoresed at 1.5 mA/tube. For electrophoresis on vertical slab gels, the acrylamide concentration was 4% in the spacer gel (pH 6.8) and 6% in the separating gel. The samples were electrophoresed until the tracking dye was 5–10 mm from the end of the gel, and the gels were fixed and stained in 40% methanol, 7% acetic acid, 0.2% Coomassie blue R-250 for 2 h (tube gels) or 30 min (slab gels) and destained by diffusion in 40% methanol, 7% acetic acid. For molecular weight determinations, the mobility of unknown proteins was compared with those of suitable protein standards. For determination of protein-bound $^3$P, the $M_r = 140,000$ and $M_r = 116,000$ Coomassie blue-stained bands were sliced from the destained gels and radioactivity was determined by scintillation spectrometry in 5 ml of ACS scintillation fluid (Amersham/Searle). Autoradiograms were made by exposing the Coomassie blue-stained slab gels to DuPont Cronex x-ray film. Protein concentration was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard. Samples and standards were routinely

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*The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PKI, cAMP-dependent protein kinase inhibitor; C subunit, catalytic subunit of cAMP-dependent protein kinase.
precipitated with cold 10% trichloroacetic acid prior to determination of protein content.

Buffers—Buffers A, B, and C (see "Results") all contained 20 mM triethanolamine HCl (pH 7.5), 20% (v/v) glycerol, 1 mM dithiothreitol, 0.02% NaN3. In addition to these ingredients, Buffer A contained 50 mM EDTA and 5 μg/ml each of the protease inhibitors leupeptin, antipain, chymostatin, and pepstatin; Buffer B contained 50 mM EDTA and 4 μg/ml each of the above protease inhibitors.

RESULTS

Rationale for Purification of Phosphorylase Kinase from the Glycogen-rich Fraction—The glycogen-rich (high speed) pellet from rat liver extracts was chosen for several reasons as the source of phosphorylase kinase for further purification. Although this fraction contains approximately 4% (15% when fully activated) of homogenate phosphorylase kinase activity, the limited number of proteins sedimenting with this fraction yields a phosphorylase kinase specific activity some 30-fold greater than that in the original homogenate and 10-fold greater than that in the high speed supernatant fluid (10). It is also known that this fraction of phosphorylase kinase is activatable by the cyclic AMP-dependent protein kinase and is stimulated by Ca2+ (11), two properties consistent with current models for the hormonal regulation of liver glycogenolysis (10). Furthermore, although considerable effort has been made to purify to homogeneity the enzyme from soluble liver extracts, no single method has proven to be satisfactory with respect to both purity of the enzyme and regulation of its activity (2, 3, 12).

Acquisition of the Glycogen Pellet—The low total amount of enzyme in the glycogen-rich fraction and the probable sensitivity of the enzyme to proteolysis (2, 12) required a large number of rats and the use of relatively gentle methods for the disruption of kilogram quantities of tissue. Thus, a total of 1000 rats of 300-600 g in body weight (male and female) were killed (30/session, 60/day) and the glycolytic enzymes obtained from the approximately 12 kg of liver were pooled and stored at -70 °C until further use. A detailed description of the acquisition of the glycogen pellet from 30 rats follows.

The rats were killed by stunning followed by dislocation of the cervical vertebrae. The liver was rapidly removed and placed in 15% glycerol at -5 °C until 30 livers (~300 g) were obtained. The livers were rinsed with fresh 15% glycerol (in Buffer A) cooled to -5 °C using a loose fitting motor-driven Teflon pestle in a glass mortar. The homogenate was then centrifuged at 8,700 x g for 30 min at -5 °C and the supernatant fluid was filtered through cheesecloth and glass wool and then centrifuged at 140,000 x g for 30 min at -5 °C using a loose fitting motor-driven Teflon pestle in a glass mortar. The yellowish, clear supernatant fluid containing about 90% of the phosphorylase kinase activity originally in the glycogen pellet was carefully removed so as not to disturb a loosely packed, brownish material overlaying the pellet. The supernatant fluid was adjusted to pH 7.2 with 1 N acetic acid and then made 5% (w/v) polyethylene glycol 8000.2 After mixing for 45 min at 4 °C, the cloudy solution was centrifuged at 24,000 x g for 30 min and the supernatant fluid was discarded.

The small pellet containing 60% of the activity of the previous fraction was dissolved in 10 ml of Buffer B and applied to a Sepharose 6B column (2.5 x 85 cm) equilibrated in Buffer C. The column was run at 22 ml/h and 4.3-ml fractions were collected. Phosphorylase kinase activity in a 20-μl aliquot of the fractions was determined in a 15-min assay. Protein was monitored at 280 nm. Fraction 41 was turbid and corresponds to the exclusion volume (V0) of this column. The fractions indicated by —— were pooled and further washed with 1 M NaCl in Buffer C; further washing with 1 M NaCl in Buffer C failed to elute additional phosphorylase kinase activity. Fraction volume was 1.3 ml and phosphorylase kinase activity was determined in a 10-min assay using 10 μl of a 4-fold dilution of the fraction. In both A and B, 15,000 cpm is equivalent to 395 pmol of phosphate incorporated.

3 Using this column under the same conditions, pure rabbit skeletal muscle phosphorylase kinase elutes as a symmetrical peak at the same volume as the liver enzyme, corresponding to Mr = 1.3 x 106.

4 Some other preparations contain material of Mr ≈ 35,000 in variable amounts, which does not apparently alter the regulatory features of the enzyme and is thus considered a contaminant. A particularly obvious example of this contaminant is seen in Fig. 6.

5 Accurate values for specific activity and total activity are difficult to determine in homogenates due to the low activity of this enzyme relative to other protein kinases which results in a high background of 32P incorporation. It is also complicated by the presence of phosphoprotein phosphatases and nucleotidases.

3 Recovery at this step can be increased by lowering the pH to 6.9 or below; however, other proteins are then precipitated which are difficult to remove in the subsequent steps.
gentle processing of the liver is especially important for the isolation of a high molecular weight activatable (by phosphorylation) form of the enzyme, presumably by limiting proteolytic activity. The activities shown in Table I were determined prior to activation by the cyclic AMP-dependent protein kinase but in the presence of 10 units/ml heparin (a stimulator of phosphorylase kinase (3)). Under optimal conditions, the activated enzyme has a specific activity of approximately 1-2 μmol·min^{-1}·mg^{-1} protein.

Purity and Tentative Subunit Composition of the Enzyme—Electrophoresis in the presence of 0.1% SDS of the purified enzyme on 6% polyacrylamide gels reveals three major peaks and several minor peaks of protein (Fig. 2, top). The two prominent peaks migrating in the Mr = 140,000 and 116,000 range are comparable in mobility to the α and β subunit of rabbit skeletal muscle phosphorylase kinase (middle). A third peak, migrating in the Mr = 45,000 region is of similar, but not identical, Mr, as the γ subunit of the skeletal muscle enzyme and this slight difference is seen with all preparations of the liver enzyme. Mixing the liver and skeletal muscle enzymes prior to electrophoresis yields protein patterns upon electrophoresis similar to those seen with either enzyme alone (Fig. 2, bottom). A fourth, low Mr, protein component (RF = 0.85), not apparent under these conditions with either the liver or skeletal muscle enzyme alone, becomes evident when the two enzymes are mixed. This component is initially clearly visible when the gels are destained but becomes less evident with storage of the gels in acetic acid. Further experiments showed that this low Mr, component of the liver and skeletal muscle enzyme co-migrated with pig brain calmodulin (not shown). By analogy with the muscle enzyme (13), it is tentatively identified as calmodulin. Thus, the subunit composition of liver phosphorylase kinase appears to be qualitatively and quantitatively similar to that of rabbit skeletal muscle. By inference from the fact that the native liver and muscle enzymes co-migrate upon gel filtration, the liver enzyme is tentatively assigned the native structure of (αβγγ), with approximate (±5%) subunit values of α, Mr = 140,000; β, Mr = 116,000; γ, Mr = 45,000; and δ, Mr = 17,500.

Catalytic Properties of the Purified Liver Enzyme—The time course of the phosphorylase kinase reaction is concave upward (Fig. 3) and is similar to that seen with the enzyme in the liver glycogen pellet (11), with that from partially purified soluble liver fractions (3) and with the nonactivated form of rabbit skeletal muscle phosphorylase kinase (14). Prior incubation with both Mg^{2+} and ATP, but not with either alone,
results in a marked increase in phosphorylase kinase activity (Table II), suggesting that the concave upward time course seen in Fig. 3 is due to activation of the enzyme by phosphorylation rather than by proteolysis during the phosphorylase kinase reaction. The presence of a contaminating phosphorylase kinase cannot be ruled out at present, although the fact that phosphorylase kinase activity at a given time point in Fig. 3 is linear with respect to enzyme amount (Fig. 3, inset) argues for an intramolecular phosphorylation, i.e. autophosphorylation. In addition, the presence of the heat stable inhibitor (PKI) of the cyclic AMP-dependent protein kinase catalytic subunit (C subunit) is without effect on either the time course of the phosphorylase kinase reaction or the MgATP-dependent activation of the enzyme (not shown).

The activity of the enzyme as isolated is maximal at pH 6.8-7.2 and drops off sharply at higher or lower pH values (Fig. 4). The sharp activity optimum at or near neutral pH is clearly different from that seen with skeletal muscle phosphorylase kinase (15) and is similar, although somewhat higher than that found for a partially purified preparation of liver phosphorylase kinase. The apparent autoactivation seen in our experience with a partially purified enzyme from soluble liver fractions (3), the pH optimum is identical with that in Fig. 4 (T. D. Chrisman and J. H. Exton, unpublished observations).

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**Table II**

**Autoactivation of rat liver phosphorylase kinase**

The 25-μl incubation mixture contained 2 μg of phosphorylase kinase, 20 mM MES (pH 6.8), and, where indicated, 3 mM Mg(C₂H₃O₂)₂ or 50 μM ATP or both. The incubation at 30 °C was initiated by the addition of H₂O or ATP and after 5 min was terminated with 75 μl of 4 °C PKI and the tubes were placed in ice H₂O. (PKI, while not relevant to this particular experiment, was included since in other experiments using the same conditions, C subunit was present. As noted elsewhere, PKI does not affect phosphorylase kinase activity.) A 10-μl aliquot was then tested in the standard phosphorylase kinase activity assay. The nonincubated control was kept in ice but otherwise treated the same as the incubated control. Activity is expressed as picomoles of phosphate incorporated in 5 min at pH 6.8.

<table>
<thead>
<tr>
<th>Time of incubation at 30 °C</th>
<th>Additions</th>
<th>Phosphorylase kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>3 mM Mg(C₂H₃O₂)₂</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>50 μM ATP</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>3 mM Mg(C₂H₃O₂)₂ + 50 μM ATP</td>
<td>60</td>
</tr>
</tbody>
</table>

**Table III**

**Activity of rat liver phosphorylase kinase against potential protein substrates**

The 50-μl reaction mixture contained 80 ng of phosphorylase kinase and 4 mg/ml each of the indicated protein substrates. Activity was determined at pH 6.8 in a 5-min incubation; other conditions were as described under "Materials and Methods." Results are expressed as percentage of activity relative to skeletal muscle phosphorylase b.

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle phosphorylase b</td>
<td>100</td>
</tr>
<tr>
<td>Rat liver phosphorylase b</td>
<td>100</td>
</tr>
<tr>
<td>Casein</td>
<td>2</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Histone, Type II-A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Histone, Type V-S</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**Fig. 4.** pH dependency of nonactivated liver phosphorylase kinase. The activity of the enzyme as isolated was determined as a function of pH. The concentration of the indicated buffers was 20 mM. Incubation time was 7 min and phosphorylase b was 4 mg/ml. Other conditions were as described under "Materials and Methods." TEA, triethanolamine.

**Fig. 5.** Ca²⁺ requirement of liver phosphorylase kinase. Activity of the enzyme as isolated was determined as a function of pCa²⁺ using CaEGTA buffers. pCa²⁺ was determined as described previously (9). Activity in a 7-min assay was determined at pH 6.8 (20 mM MES) using 2 mg/ml phosphorylase b. Other conditions were as described under "Materials and Methods."
at pH 6.8 and above is not apparent at pH 5.9 (not shown) as evidenced by a linear (rather than concave upward) time course of product formation, and in part accounts for the decreased activity of the enzyme at low pH. A decreased formation of the MgATP complex (accompanied by an increase in free Mg$^{2+}$ and in free ATP) at low pH may also have an effect on enzyme activity.

**Protein Substrates**—The activity of the enzyme against other potential protein substrates is low when compared to phosphorylase b (Table III). Under the standard assay conditions, liver and muscle phosphorylase b serve equally well as phosphate acceptors, whereas histone (Types II-A and V-S) and phosvitin serve very poorly, if at all, as protein substrates. Casein can serve as a substrate although to a much lesser degree than phosphorylase b. It is not clear at this time if this casein kinase activity represents an enzyme separate from phosphorylase kinase or, as with the skeletal muscle enzyme (14), reflects some intrinsic casein kinase activity of phosphorylase kinase. Preliminary experiments show this casein kinase activity to be resistant to inhibition by EGTA and to stimulation by heparin.

Rabbit skeletal muscle glycogen synthase does not serve as a substrate when present at 0.25 mg/ml (for this experiment phosphorylase b was also at 0.25 mg/ml) at the phosphorylase kinase amounts normally used. However, some phosphorylation was evident when the amount of phosphorylase kinase was increased 10-fold, and this phosphorylation was inhibited (90%) by 0.5 mM EGTA. Liver glycogen synthase has not been tested as a protein substrate.

**Ca$^{2+}$ Requirement**—The apparent Ca$^{2+}$ requirement (as determined in the presence of EGTA) of the purified enzyme (Fig. 5) is essentially the same as that seen for the enzyme in crude liver extracts (1, 16). The enzyme is stimulated by low concentrations of free Ca$^{2+}$ which are within the range reported for hepatocytes (17) and stimulation by Ca$^{2+}$ is maximal in the 10$^{-7}$ to 10$^{-6}$ M range, with half-maximal activity seen at about 5 x 10$^{-8}$ M. The inhibition by Ca$^{2+}$ at >10$^{-6}$ M is characteristic of the liver enzyme (1, 18, 19), although the

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Fig. 6. Incorporation into the Mr = 140,000 and 116,000 components of liver phosphorylase kinase. Polyacrylamide gel electrophoresis was performed under the conditions described in Materials and Methods. The Mr = 140,000 component was incubated at 30 °C for 5 min in a total volume of 55 μl containing 0.1% SDS, 5.2 × 10^5 cpm of [γ-32P]ATP, 1% glycerol, 10 mM triethanolamine, HCl (pH 7.5), and where indicated 1000 units of C subunit (2 μg). The reaction was initiated with Mg(CH$_2$CO$_2$)$_2$-ATP-[γ-32P]ATP and terminated with 0.2 ml of cold 10% trichloroacetic acid, 10 mM ATP. The samples were then prepared for gel electrophoresis as described under "Materials and Methods." Lanes A and B are Coomassie blue-stained protein patterns of 10 μg of skeletal muscle phosphorylase kinase and 1.5 μg of liver phosphorylase kinase, respectively. The lowest band (Mr = 35,000) in lane B is a contaminant in this preparation of the enzyme. Lanes C and D are autoradiograms of liver phosphorylase kinase incubated in the absence and in the presence of C subunit, respectively.

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Fig. 7. Activation and phosphorylation of liver phosphorylase kinase. Activation of phosphorylase kinase (A and C) was carried out at 30 °C. The 40-μl incubation mixture contained 1.8 mg of phosphorylase kinase, 20 mM MES (pH 6.8), 3 mM Mg(CH$_2$CO$_2$)$_2$ 50 μM ATP, 0.5% glycogen. Where indicated, the incubation mixture also contained 50 units of C subunit. The reaction was initiated with Mg(CH$_2$CO$_2$)$_2$-ATP and terminated at the indicated times with 0.36 ml of cold PKI. 10 μl aliquots were then assayed for phosphorylase kinase activity at pH 7.5 as described under "Materials and Methods." Phosphorylation of phosphorylase kinase (B and D) and polyacrylamide gel electrophoresis in vertical slab gels were carried out as described for Fig. 6. Where indicated, 75 units of C subunit were present during the incubation. After destaining the polyacrylamide gels, radioactivity in the Mr = 140,000 and 116,000 components was determined as described under "Materials and Methods."
"Materials and Methods." The buffers were 20 mM MES (O, ●), 20 mM triethanolamine HCl (△), and 20 mM Tris (□, ■).

mechanism of this inhibition is not known.

Phosphorylation and Activation—As pointed out above, the concave upward time course of the phosphorylase kinase reaction (Fig. 2) and the MgATP-dependent activation of the enzyme (Table II) suggest that the enzyme is activated by phosphorylation. Incubation of the enzyme with Mg<sup>2+</sup> and [γ-<sup>32</sup>P]ATP resulted in the incorporation of 32 p into the phosphorylation. Incubation of the enzyme with Mg<sup>2+</sup> and the concave upward time course of the phosphorylase kinase closely resembles the muscle enzyme as to subunit composition, one possible exception being a somewhat larger subunit. The presence of a component of identical mobility on SDS gel electrophoresis to the subunit of the muscle enzyme, which has been identified for the muscle enzyme as calmodulin (13), suggests that this Ca<sup>2+</sup>-binding protein also plays a role in the Ca<sup>2+</sup> sensitivity of the liver enzyme. However, the greater sensitivity of the liver enzyme to Ca<sup>2+</sup>, the marked inhibition by higher than optimal concentrations of Ca<sup>2+</sup> (>5 × 10<sup>-6</sup> M), and the incomplete inhibition by EGTA suggest that the interaction of Ca<sup>2+</sup> with liver phosphorylase kinase differs from other phosphorylase kinases. Another similarity evident from phosphorylation studies is that, like the mammalian muscle enzyme, the M<sub>r</sub> = 140,000 and 116,000 (β) protein components exclusively (Fig. 6, lane C) and this incorporation was further increased in the presence of C subunit<sup>6</sup> (Fig. 6, lane D). The time course of phosphorylation in the presence and absence of C subunit were correlated with activation of the enzyme (Fig. 7). Further studies will be required in order to establish the relationship of phosphorylation of a given component to the activation of the enzyme.

Effect of Activation on pH Optimum and Ca<sup>2+</sup> Requirement—Activation by phosphorylation is most evident at pH values between 7 and 8 (Fig. 8) with an apparent inhibition of activity seen at pH values below 6.5. These effects were reproducible and are similar to those seen with a partially purified form of the enzyme (2). The activated enzyme was unchanged with respect to the Ca<sup>2+</sup> requirement and inhibition by EGTA (not shown).

DISCUSSION

The purification of rat liver phosphorylase kinase by the method described above is rapid, relatively simple, and provides a near homogeneous preparation of an enzyme having the regulatory features consistent with its presumed role in the regulation of liver glycogenolysis. Some aspects of the purification method deserve special comment. The rapid removal and chilling of the liver and the use of cold (<0 °C) buffers containing glycerol and several protease inhibitors coupled with the gentle homogenization of the tissue allow the subsequent isolation of an enzyme which has the properties described above. This approach apparently reduces proteolytic activity both directly (protease inhibitors) and indirectly (low temperature) and stabilizes the enzyme (glycerol is more effective than sucrose in all steps of purification). Omission of any of the above components results in the generation of variable amounts of a low molecular weight form of the enzyme similar to that seen with other methods.

<sup>6</sup>The phosphorylated component of M<sub>r</sub> = 40,000 in lane D is due to the autophosphorylation of C subunit (20).

of purification (1). A second aspect of the purification procedure is the use of the enzyme initially associated with the glycogen-rich fraction. It was recently pointed out that, although the total phosphorylase kinase activity is low in this fraction when compared to initial extracts, the specific activity is relatively high (10) and enzyme activity is sensitive to regulation by both phosphorylation and Ca<sup>2+</sup> (11). The physical nature of the association of the enzyme with this fraction is not known, although in muscle, some phosphorylase kinase is associated with the glycogen particle (21). Finally, the use of immobilized heparin as a purification component stems from studies of the effects of free heparin on the activities of various protein kinases (22, 23) and, especially, the finding that heparin is a potent stimulator of muscle (24) and liver (3) phosphorylase kinases. Based on these observations, heparin-Sepharose CL-6B was tested and was found to be a useful tool in the purification procedure.

The initial findings presented here suggest that liver phosphorylase kinase closely resembles the muscle enzyme as to subunit composition, one possible exception being a somewhat larger subunit. The presence of a component of identical mobility on SDS gel electrophoresis to the subunit of the muscle enzyme, which has been identified for the muscle enzyme as calmodulin (13), suggests that this Ca<sup>2+</sup>-binding protein also plays a role in the Ca<sup>2+</sup> sensitivity of the liver enzyme. However, the greater sensitivity of the liver enzyme to Ca<sup>2+</sup>, the marked inhibition by higher than optimal concentrations of Ca<sup>2+</sup> (>5 × 10<sup>-6</sup> M), and the incomplete inhibition by EGTA suggest that the interaction of Ca<sup>2+</sup> with liver phosphorylase kinase differs from other phosphorylase kinases. Another similarity evident from phosphorylation studies is that, like the mammalian muscle enzyme, the M<sub>r</sub> = 140,000 and 116,000 components of the liver enzyme are readily phosphorylated by the cyclic AMP-dependent protein kinase and this phosphorylation parallels activation of the enzyme. The above findings, taken with identical migrations on gel filtration of the native liver and muscle phosphorylase kinases, suggest that liver phosphorylase kinase is of the structure (αβ,γδ,ε). Clearly, further studies are required before this tentative structure can be definitely assigned to the liver enzyme.

The availability of highly purified liver phosphorylase kinase will permit for the first time a clear and unambiguous determination of the subunit structure and the regulatory features of the enzyme. This is particularly relevant to the study of hormonal regulation of liver glycogenolysis for two reasons: first, due to a lack of pure liver phosphorylase kinase, many hypotheses concerning the role of phosphorylase kinase in liver glycogenolysis are based heavily on analogy to the rabbit skeletal muscle enzyme and may well be misleading, and second, reconstruction of the known enzymatic machinery of glycogen metabolism should yield some insight into the relative importance of phosphorylase kinase and cyclic AMP-dependent protein kinase in determining the activities of phosphorylase and glycogen synthase, the ultimate targets of hormonally altered glycogen metabolism.

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