Phosvitin Kinase from the Liver of the Rooster

PURIFICATION AND PARTIAL CHARACTERIZATION*

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SUMMARY

Phosvitin kinase, the enzyme that phosphorylates the egg yolk protein phosvitin, has been purified more than 5000-fold from the supernatant of rooster liver, a tissue that synthesizes phosvitin only after estrogen administration. The purified enzyme prepared from the estrogen-treated liver was recovered in high yield (49%) and was very stable at 0-4°C. It sedimented in sucrose as a single peak with a S20,w of approximately 7.6. Preliminary studies indicated that the native enzyme consists of several subunits with the major component having a molecular weight between 40,000 and 50,000.

Rooster liver phosvitin kinase phosphorylated phosvitin at a 10-fold higher rate than it phosphorylated casein. It did not phosphorylate histone or protamine. Mg2+ stimulated the rate of phosphorylation, while Co2+ and Mn2+ were inhibitory. Phosvitin kinase activity was independent of cyclic adenosine 3':5'-monophosphate. A unique property of the enzyme was its ability to utilize GTP as well as ATP as a phosphate donor for phosphorylation of phosvitin.

Despite the fact that estrogen administration to roosters led to a marked induction in the hepatic synthesis of phosvitin, there was no significant difference in the phosvitin kinases prepared from treated and untreated livers in either the specific activity or the apparent Km for ATP (7.7 μM). These data suggest that phosphorylation of phosvitin by phosvitin kinase is probably not a rate-limiting event in the overall process by which estrogen induces specific protein synthesis in rooster liver.

Phosvitin is one of the two major egg yolk proteins normally synthesized in the liver of the laying hen and transported in the blood to the developing oocyte (1, 2). Phosvitin is not normally made in the livers of roosters, but its hepatic synthesis, together with that of a very low density lipoprotein, can be induced by the administration of estrogen (3-5). Since the rooster has no ovary to remove these yolk proteins, phosvitin and very low density lipoprotein accumulate in high levels in the plasma of the estrogen-treated rooster (6). Phosvitin is a unique protein in that greater than 50% of its amino acid composition consists of serine residues, almost all of which are phosphorylated (7). This unusual structural feature of phosvitin provides an invaluable marker for its identification and makes the study of its in vitro synthesis in cell-free rooster liver preparations a useful biological system for characterizing the action of estrogen on specific gene expression.

One of the requirements for phosvitin synthesis may be the presence of a specific protein kinase to attach the phosphate residues to the serine moieties. A class of protein kinases (EC 2.7.1.37) that preferentially catalyzes the transfer of the terminal phosphate from ATP to the serine residues of phosvitin has been identified in a variety of animal tissues that do not contain phosvitin, such as rat liver (8); rabbit mammary gland (9); calf brain (10); calf uterus (11); rabbit reticulocytes (12); human erythrocyte membranes (13); ox brain (14); mammalian skin, muscle, and connective tissue (15); rat kidney (16); brewer's yeast (10); amphibian ovary (17); and fish roe (18). Although the physiological role of these non-avian phosphorylation reactions is not known, these previously described phosvitin kinases appear to be a functionally different type of enzyme than the cyclic AMP-dependent protein kinases that participate in mammalian hormone action (19).

Despite the fact that phosvitin is a major synthetic protein of the liver of the laying hen and the estrogen-treated rooster and despite the recent interest in phosphorylation reactions in relation to hormone action, the phosvitin kinase enzyme from the estrogen-stimulated avian liver does not appear to have been previously studied. In this paper we describe the purification and characterization of some of the properties of rooster liver phosvitin kinase, the enzyme that phosphorylates phosvitin in vitro.

MATERIALS AND METHODS

Chemicals

[γ-32P]ATP (14.6 to 31 Ci per mmole) and [γ-32P]GTP (14 Ci per mmole) were purchased from New England Nuclear Corp. 17β-Estradiol, phosvitin, casein, calf thymus histone (type I) The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate.
II-A), salmon protamine, bovine serum albumin, blue dextran, rabbit muscle aldolase, sucrose (RNase-free), ammonium sulfate, toluidine blue, and all nucleosides and nucleotides were purchased from Sigma Chemical Co. Sodium dodecyl sulfate was obtained from Pierce Chemical Co. Denatured benzaldehyde (purified) was purchased from Fischer Scientific Laboratories. Apoferritin, bovine liver catalase, cytochrome c, chymotrypsigenin, ovalbumin, human γ-globulin, and myoglobin were purchased from Schwarz-Mann. Bio-Gel 10% agarose A-0.5M (200 to 400 mesh) and Bio-Gel 6% agarose A-5M (200 to 400 mesh) were purchased from Bio-Rad Laboratories. Coomassie brilliant blue was obtained from K & K Laboratories, Plainview, N. Y. Cellulose phosphate P-11 (Whatman) was obtained from H. Reeve Angel, Inc.

**Animals**

White Leghorn roosters, weighing 2.0 to 2.3 kg and injected intramuscularly with either benzyl benzaldehyde or 17-β-estradiol dissolved in benzyl benzaldehyde, were the source of all tissues used.

**Determination of Plasma Phosphoprotein Content**

Heparinized blood, collected from the wing vein of roosters, was centrifuged at 1000 rpm for 15 min at 24°C, the plasma was separated (average volume obtained from each rooster was 3 ml), and the proteins in plasma were precipitated with 4 volumes of cold 20% (w/v) trichloroacetic acid. The resulting precipitate was resuspended in 2 ml of 5% trichloroacetic acid, heated at 90° for 20 min, and centrifuged at 1000 rpm for 15 min. Liquid was removed from the protein precipitate by successive washings with 2 ml of hot ethanol; 4 ml of chloroform ether-etheranol (v/v/v) 1:2:2; 2 ml of acetone; and 2 ml of ether. The level of phosphoprotein in plasma was then determined by measurement of the alkali-labile phosphorus content of the lipid-free protein precipitation. This procedure, which is highly specific for phosvitin in plasma (1, 5), consisted of the following steps. One milliliter of 1 N NaOH was added to each lipid-free plasma protein precipitate, the mixture was boiled 15 min, the protein was reprecipitated with 2 ml of 20% trichloroacetic acid and collected by centrifugation at 1000 rpm for 10 min, and the content of phosphate in the resulting supernatant was determined by the method of Fiske and SubbaRow (20).

**Phosvitin Kinase Assay**

The activity of phosvitin kinase was determined by measurement of the enzymatic transfer of 32P from (γ-32P)ATP to phosvitin that was partially dephosphorylated. The standard assay (unless otherwise stated) consisted of incubation of the following components in a final volume of 0.1 ml: 50 mM potassium phosphate, pH 6.6, 7.5 mM MgCl₂, 100 µg of phosvitin (50% dephosphorylated), 600 pmoles of (γ-32P)ATP containing 2.4 to 4.8 × 10⁶ cpm, and phosvitin kinase. Incubation was carried out at 37°C, for the indicated time, and the reaction was terminated by the addition of 2 ml of cold 10% trichloroacetic acid. The mixture was allowed to stand at 0°C for 10 min, and the precipitate was collected on a Millipore filter (HAWP 0.25 µ) that was washed with 10 ml of 5% trichloroacetic acid, dried at 70° for 10 min, placed in a vial with 10 ml of solution containing 0.6% (w/v) 2,5-diphenoxazol (PPO) and 0.005% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPPO) in toluene, and counted in a liquid scintillation counter. The blank for assays containing purified enzyme preparations (Fractions III and IV) consisted of the amount of 32P incorporated into phosvitin at zero time. The blank for assays containing the less pure enzyme preparations (Fractions I and II) consisted of the amount of 32P incorporated into the endogenous proteins as determined in parallel reactions run in the absence of phosvitin for the duration of the incubation at 37°C.

**Purification of Phosvitin Kinase**

**Step I**—Roosters were given single intramuscular injections of 30 mg of 17-β-estradiol dissolved in 0.8 ml of benzyl benzaldehyde, and 4 days later the livers were removed and placed in cold buffer containing 50 mM Tris-Cl, pH 7.4, 50 mM KCl, 5 mM 2-mercaptoethanol, and 1 mM MgCl₂ (Buffer A). All subsequent steps were performed at 0–4°C. The livers were diced into small pieces with a scissors and then homogenized with 4 volumes of Buffer A in a Waring Blender for 30 to 60 s at full speed. The homogenate was centrifuged at 1000 × g for 15 min, followed by centrifugation at 26,000 × g for 30 min. The supernatant (S-26) was then centrifuged at 105,000 × g for 90 min, and the resulting supernatant (S-105) was designated Fraction I. Its protein concentration was 21.3 mg/ml.

**Step II**—A 0 to 60% ammonium sulfate fraction was prepared from Fraction I by addition of 1.5 volumes of a saturated solution of ammonium sulfate buffered in 0.1 M Tris-Cl, pH 7.4. The precipitated protein (Fraction II, 222 mg) was suspended in 10 ml of buffer containing 20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (Buffer B).

**Step III**—Ten milliliters of Fraction II were applied to a column (90 × 2.5 cm) packed with Bio-Gel 10% agarose A-0.5M (200 to 400 mesh) equilibrated in buffer B. The column was eluted with Buffer B at a flow rate of 50 ml per hour, and 5 ml fractions were collected. Phosvitin kinase was identified in tubes 44 to 50 (Fraction III).

**Step IV**—Fraction III (66.1 mg) was dialyzed against buffer containing 50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 5 mM 2-mercaptoethanol, and 0.1 M EDTA (Buffer C) and then applied to a cellulose phosphate P-11 column (6.1 × 1.5 cm) equilibrated with Buffer C. Fractions of 2.5 ml each were collected by 50 ml of Buffer C at a flow rate of 50 ml per hour, followed by 130 ml of a linear gradient of NaCl (0.5 to 1.2 M), followed by 25 ml of Buffer C containing 1.2 M NaCl. The tubes containing phosvitin kinase activity, eluting at 0.9 M NaCl, were pooled (Fraction IV), concentrated by membrane pressure filtration (Amicon Co., Lexington, Mass.), and dialyzed against buffer containing 50 mM Tris-Cl, pH 7.4, 0.2 M NaCl, 5 mM 2-mercaptoethanol, and 0.1 M EDTA (Buffer D). Aliquots of Fractions I, II, and III were stored at −190°C. Fraction IV was stored at 0–4°C. Additional details of the purification of phosvitin kinase are given in the legends to Figs. 2 and 3 and to Table I.

The protein content of each fraction was determined by a modification of the method of Lowry et al. (21) using bovine serum albumin as a standard.

**Density Gradient Centrifugation**

The sedimentation coefficient of phosvitin kinase was determined by the procedure of Martin and Ames (22), in which a comparison of its rate of sedimentation in sucrose gradients was made with that of proteins of known S values. Linear gradients from 5 to 20% (w/v) sucrose contained 50 mM Tris-Cl, pH 7.4, 0.2 M NaCl, 5 mM 2-mercaptoethanol, and 0.1 M EDTA. Samples (0.2 ml), containing phosvitin kinase, standard protein markers, or both, were layered over 5 ml of sucrose gradients and centrifuged for the indicated time at 4°C in a Beckman SW 6301 rotor with Spinco model L2-65B ultracentrifuge. At the end of the centrifugation, the bottom of the tube was punctured...
utilizing a Beckman Fraction Recovery System, and 8-drop fractions were collected and assessed for enzyme activity and protein content as described in the legend to Figs. 4 and 5.

**Polyacrylamide Gel Electrophoresis**

Samples were prepared according to the method of Maizel (23) as described in the legends to Figs. 6 and 9 and were run in the presence of 0.1% sodium dodecyl sulfate and 0.5 mM Tris-glycine, pH 8.2, on 13% polyacrylamide gels with a ratio of acrylamide to bisacrylamide of 37:1. The methods for staining, destaining, and counting of radioactivity of the gels is described in the legends to Figs. 6 and 9. Molecular weights were estimated by the method of Weber and Osborn (24).

**RESULTS**

**Time Course of Estradiol-induced Appearance of Phosvitin in Plasma**—The content of phosphoprotein in the plasma of untreated roosters averaged about 2 μg per ml. As previously shown in other laboratories (4–6, 25, 26), the administration of estradiol to roosters resulted in a marked increase in the plasma phosphoprotein level (Fig. 1). The rate and extent of this rise was dose-related (Fig. 1). The highest level of plasma phosphoprotein (360 μg per ml) was observed in roosters that had received an initial injection of either 30 or 60 mg of estradiol on Day 0 and a repeat injection of the same dose on Day 12. The administration of daily doses of 30 mg of estradiol for 12 consecutive days did not result in any higher levels of plasma phosphoprotein. Since the level of alkali-labile phosphorus in plasma proteins represents a reliable index of the concentration of plasma phosvitin (4, 5) and since the inorganic phosphate content of plasma is 10.4% by weight (7), it can be estimated that the maximal concentration of phosvitin in plasma resulting from the administration of pharmacological amounts of estradiol was about 200 mg per dl.

**Purification of Phosvitin Kinase**—In order to study the properties and characteristics of the liver enzyme catalyzing the phosphorylation of phosvitin, it is essential to separate it from other enzymes that incorporate 32P into protein. The starting material for the purification of rooster liver phosvitin kinase was the 105,000 × g supernatant (Fraction I). This was prepared from the livers of animals which had received a single 30-mg injection of estradiol 3 days earlier. An analysis of the time course of appearance of phosvitin in plasma following estradiol treatment (Fig. 1) indicated that the rate of hepatic synthesis and phosphorylation of phosvitin were maximal at about 4 days after hormone administration. Because of the high background incorporation of 32P from [γ-32P]ATP into endogenous proteins, assay of the crude extract was unreliable. Thus, a detailed study of the subcellular distribution of enzyme activity in crude extracts was not possible. However, the only subcellular fraction containing significant amounts of phosvitin-dependent kinase activity appeared to be the 105,000 × g supernatant fraction (Fraction I). No phosvitin-dependent activity in significant excess of that due to endogenous 32P incorporation was detected in the 900 × g pellet (crude nuclei), the 28,000 × g pellet (mitochondrial fraction), or in the 106,000 × g pellet (microsomal fraction).

When Fraction I was treated with ammonium sulfate, all the phosvitin-dependent kinase activity was contained in the fraction which precipitated at 0 to 60% (Fraction II). The 60 to 80% fraction was devoid of phosvitin kinase activity. Although only a small percentage of the total protein was removed by this procedure, the ammonium sulfate precipitation eliminated most of the endogenous phosphorylation that occurred in the preparation when assayed in the absence of added phosvitin. Following ammonium sulfate precipitation, Fraction II was chromatographed on 10% agarose and all the phosvitin-dependent kinase activity appeared as a single peak in the void volume of the column (Fraction III) (Fig. 2). Fraction III was further purified by cellulose phosphate column chromatography (Fig. 3). Virtually all of the protein in Fraction III was eluted at 0.5 M NaCl, whereas phosvitin kinase was eluted at 0.9 M NaCl (Fraction IV). The addition of the proteins eluting at 0.5 M NaCl to Fraction IV neither stimulated nor inhibited its phosvitin kinase activity.

A summary of the purification of rooster liver phosvitin kinase is given in Table I. Phosvitin kinase was purified more than 8000-fold with 49.3% cumulative recovery of activity. When the purified enzyme (Fraction IV) was rapidly frozen in a Dry Ice-acetone bath, about 30% of initial activity was lost. The purified enzyme could be stored at 4° for at least 4 months without loss of activity in a buffer containing 50 mM Tris-Cl, pH 7.4, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and either 10% glycerol, 0.9 M NaCl, or 5 mg per ml of bovine serum albumin.

**Physical Properties of Phosvitin Kinase**—Phosvitin kinase sedimented as a single peak as assessed by density gradient centrifugation (Fig. 4). Its sedimentation coefficient, determined using myoglobin, bovine serum albumin, aldolase, catalase, and apoferritin as markers, was approximately 7.6 S. This finding suggested that the native enzyme possessed a molecular weight of approximately 160,000, assuming that it is a typical globular protein. However, the behavior of the enzyme on gel filtration was anomalous, in that by filtration on both Sephadex G-200 and 10% agarose columns the purified enzyme eluted in the void volume and on a 5% agarose column it eluted as two peaks: a minor peak that co-eluted with dextran blue just after the void volume and a major peak that eluted ahead of catalase, a marker protein with a molecular weight of 250,000 (27) (data not shown). These data would suggest that phosvitin kinase may not be a typical globular protein. When purified phos-
Phosvitin kinase was prepared as described under "Materials and Methods." At each major step in the purification, aliquots of activity were obtained, each was dialyzed against buffer D, and in the same study each was assayed for phosvitin kinase activity under the standard conditions at 37° for 15 min. The protein concentration of each fraction was determined by the method of Lowry et al. (21) against a buffer blank.

Fig. 4 (right). Density gradient centrifugation of phosvitin kinase. Phosvitin kinase, 0.2 ml (50 μg), was layered over 5 to 20% sucrose gradients prepared as described under "Materials and Methods" and centrifuged for 13 hours at 48,000 rpm at 4°. The sedimentation position of phosvitin kinase (●-●) was determined by assay of 25-μl aliquots of each fraction under the standard conditions at 37° for 20 min. The amount of 32P incorporated into phosvitin without addition of column fractions (2.3 pmol) was subtracted from all values.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Total activity</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>units/mg</td>
<td>-fold</td>
<td>units</td>
</tr>
<tr>
<td>I. S-105</td>
<td>311</td>
<td>311</td>
<td>0.057</td>
<td>1.0</td>
<td>17.7</td>
</tr>
<tr>
<td>II. Ammonium sulfate, 0 to 60%</td>
<td>311</td>
<td>222</td>
<td>0.17</td>
<td>3.0</td>
<td>37.7</td>
</tr>
<tr>
<td>III. 10% agarose</td>
<td>311</td>
<td>222</td>
<td>0.39</td>
<td>6.8</td>
<td>21.9</td>
</tr>
<tr>
<td>IV. Cellulose phosphate</td>
<td>311</td>
<td>0.04</td>
<td>466.2</td>
<td>8170</td>
<td>18.6</td>
</tr>
</tbody>
</table>

Purification of phosvitin kinase

Phosvitin kinase was subjected to disc gel electrophoresis in the presence of sodium dodecyl sulfate (23, 24), a single major component plus several minor bands were seen (Fig. 5). Estimation of the molecular weight of the major component, using cytochrome c, chymotripsinogen, ovalbumin, bovine serum albumin, and the two γ-globulin chains as standards (24), gave a value between 40,000 and 50,000. This would suggest that the native phosvitin kinase may be composed of several subunits. A band identical with the major band given by the purified enzyme (Fraction IV) was seen in the less pure preparations (Fractions II and III) (Fig. 5).

Conditions of Standard Assay—The amount of phosvitin phosphorylated by the purified preparation of phosvitin kinase was linear with time (Fig. 6A), enzyme protein concentration (Fig. 6B), and phosvitin concentration under standard assay conditions. In the absence of added phosvitin the purified enzyme preparation did not incorporate any detectable 32P into endogenous proteins (Fig 6, A and B). The phosvitin used as substrate in these studies was approximately 50% dephosphorylated, as determined by measurement of its alkali-labile inorganic phosphate content (5).

Characterization of Enzymatically Phosphorylated Phosvitin—The 32P-labeled reaction products obtained by phosphorylation with phosvitin kinase under the standard assay conditions were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 7). The single major peak of radioactivity
FIG. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of phosvitin kinase preparations. Proteins were denatured and reduced by incubation in 30 mM P, (pH 6.7), 1% sodium dodecyl sulfate, 10% glycerol, and 0.1% 2-mercaptoethanol at 100° for 2 min. Fifty-microliter samples of Fractions II (shown at left), III (middle), and IV (right), each containing 0.02 to 0.05 unit of phosvitin kinase activity, were applied to 13% gels containing 0.1% sodium dodecyl sulfate and subjected to electrophoresis at room temperature at 2 ma per tube using bromophenol blue as the tracking dye. Gels were stained at 37° for 2 hours in 0.2% Coomassie brilliant blue in 50% methanol and 7% acetic acid. Destaining was done electrophoretically (24) in a solution of 5% methanol and 7% acetic acid.

that was obtained corresponded to the position of migration of authentic phosvitin identified by staining with toluidine blue. These data indicated that the measurement of 32P incorporation in the standard assay reflected a measurement of phosphorylation of phosvitin.

pH Optimum and Divalent Cation Requirement—Both pH and the concentration of divalent cation influenced the rate of phosphorylation of phosvitin. Under the standard assay conditions at 7.5 mM Mg2+, phosvitin kinase showed high activity over a broad pH range between 6 and 8 with maximal activity at pH 6.6. The increased rate of phosphorylation at pH 6.6 was not due to activation of 32P incorporation into the endogenous proteins of the enzyme preparation. Phosphate ion had no effect on enzyme activity as indicated by the data that at pH 7 rates of phosphorylation were the same whether or not potassium phosphate, 2-(N-morpholino)ethanesulfonate, or Tris-Cl was used as the buffer (data not shown).

The effect of varying concentrations of Mg2+, Co2+, and Mn2+ on rates phosphorylation of phosvitin is shown in Fig. 8. 

Effect of Nucleosides and Nucleotides—The effects of various nucleosides and nucleotides on the activity of phosvitin kinase were compared (Table II). All nucleosides and nucleotides were tested at a final concentration that was 100-fold higher than that of the [γ-32P]ATP. None of these agents, notably cyclic AMP, stimulated phosvitin kinase activity. The most potent inhibitor of enzyme activity was ADP, suggesting that a reversibility of 32P transfer between phosvitin and ADP was occurring, as has previously been shown for phosvitin kinase isolated from calf brain and yeast (10). The next most potent inhibitor of rooster liver phosvitin kinase activity was GTP. This finding suggested the possibility that GTP might be competing with ATP as a substrate for the phosvitin kinase by providing the γ-phosphate for enzymatic transfer. Therefore, experiments were carried out to compare the efficacy of [γ-32P]ATP and [γ-32P]GTP to serve as phosphate donors for phosvitin kinase (Table III). [γ-32P]GTP proved to be an effective phosphate donor for this reaction, but the rate of phosphorylation of phosvitin with [γ-32P]GTP as a substrate was approximately one-half that with [γ-32P]ATP.
serum albumin were not phosphorylated by phosvitin kinase in the presence and absence of 0.1 mM cyclic AMP), and bovine protamine, calf thymus histone (tested either pH 6.6 (Fig. 9) or pH 7.4 (data not shown).

However, this possibility was not tested directly.

Substrate Specificity—The ability of rooster liver phosvitin kinase to phosphorylate a variety of protein substrates was evaluated (Fig. 9). Of all the proteins tested only phosvitin and casein were phosphorylated by the enzyme. That the unique structural sequence is the recognition site for the phosvitin kinase enzyme. Protamine, calf thymus histone (tested with authentic phosvitin, which was identified by staining with 5 mg/100 ml of toluidine blue (top).

The inhibition of phosvitin kinase activity by ITP suggested that ITP, like GTP, could serve as a substrate for this reaction. However, this possibility was not tested directly.

**TABLE II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Kinase activity (pmoles ³P incorporated/10 min)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>42.3</td>
<td>100</td>
</tr>
<tr>
<td>+ Adenosine</td>
<td>42.2</td>
<td>100</td>
</tr>
<tr>
<td>+ AMP</td>
<td>40.8</td>
<td>96</td>
</tr>
<tr>
<td>+ Cyclic AMP</td>
<td>41.3</td>
<td>98</td>
</tr>
<tr>
<td>+ ADP</td>
<td>3.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>43.5</td>
<td>100</td>
</tr>
<tr>
<td>+ Guanosine</td>
<td>43.6</td>
<td>100</td>
</tr>
<tr>
<td>+ GMP</td>
<td>40.0</td>
<td>92</td>
</tr>
<tr>
<td>+ Cyclic GMP</td>
<td>47.1</td>
<td>108</td>
</tr>
<tr>
<td>+ GDP</td>
<td>18.2</td>
<td>42</td>
</tr>
<tr>
<td>+ GTP</td>
<td>10.1</td>
<td>23</td>
</tr>
<tr>
<td>+ CMP</td>
<td>46.0</td>
<td>106</td>
</tr>
<tr>
<td>+ CDP</td>
<td>43.8</td>
<td>101</td>
</tr>
<tr>
<td>+ CTP</td>
<td>44.3</td>
<td>102</td>
</tr>
<tr>
<td>+ UMP</td>
<td>42.6</td>
<td>98</td>
</tr>
<tr>
<td>+ UDP</td>
<td>39.3</td>
<td>90</td>
</tr>
<tr>
<td>+ UTP</td>
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<tr>
<td>Experiment C</td>
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<tr>
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<tr>
<td>+ TTP</td>
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<td>42</td>
</tr>
<tr>
<td>+ TDP</td>
<td>52.8</td>
<td>83</td>
</tr>
<tr>
<td>+ TTP</td>
<td>53.6</td>
<td>85</td>
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</table>

* Cyclic GMP, cyclic guanosine 3'-5'-monophosphate.
Comparison of Liver Phosvitin Kinase of Control and Estradiol-treated Roosters—The administration of estradiol to roosters is known to cause an induction in the hepatic synthesis of phosvitin (3–5). In order to determine whether or not phosphorylation might be a rate-limiting event in estrogen action, it became of interest to compare phosvitin kinase from livers of untreated and estradiol-treated roosters. Although estradiol caused a 2.5-fold increase in liver weight and a 100-fold increase in the content of plasma phosphoprotein, there was no appreciable change in the specific activity of phosvitin kinase prepared from the livers of treated and untreated animals (Table IV). The rise in total content of phosvitin kinase activity in the liver of the treated animal was of the same order of magnitude as the increase in liver weight.

The $K_m$ for ATP and the $K_m$ for phosvitin and the reaction $V_{max}$ for these two substrates were determined for both the untreated and treated preparations of enzyme. Under the conditions of the standard assay, the apparent $K_m$ for ATP was 7.7 μM for both enzyme preparations. However, the estradiol-treated enzyme had an apparent $K_m$ for phosvitin that was 3.3-fold lower than that of the untreated enzyme (Fig. 10). The reason for this difference in $K_m$ is not known. No difference in $V_{max}$ of the two enzymes was noted for either substrate.

### Table III

Comparison of $[\gamma-\text{P}]$ATP and $[\gamma-\text{P}]$GTP as phosphate donors for phosvitin kinase

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>Kinase activity (pmoles $\text{P}^3$ incorporated/15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\gamma-\text{P}]$ATP</td>
<td></td>
</tr>
<tr>
<td>- Phosvitin</td>
<td>0.8</td>
</tr>
<tr>
<td>+ Phosvitin</td>
<td>169.5</td>
</tr>
<tr>
<td>$[\gamma-\text{P}]$GTP</td>
<td></td>
</tr>
<tr>
<td>- Phosvitin</td>
<td>0.7</td>
</tr>
<tr>
<td>+ Phosvitin</td>
<td>85.5</td>
</tr>
</tbody>
</table>

### Table IV

Comparison of phosvitin kinase activity of livers from control and estradiol-treated roosters

Four days after a single intramuscular injection (0.8 ml) of either benzyl benzoate (control) or 30 mg of 17-β-estradiol dissolved in benzyl benzoate (estradiol-treated), roosters were killed and their plasma and livers were obtained for measurement of phosphoprotein content and phosvitin kinase activity, respectively, as described under "Materials and Methods." Each enzyme reaction was incubated at 37° for 15 min under the standard conditions and contained 12 μg of phosvitin kinase, 0.1 mM cyclic AMP as indicated, and 100 μg of one of the following substrates: ▲—▲, phosvitin; ●—●, casein; ■—■, protamine, calf thymus histone (type II-A), bovine serum albumin, or none. The amount of $\text{P}^3$ incorporated at zero time (1.6 pmoles) was subtracted from all values.

### DISCUSSION

The present studies report the purification and partial characterization of the properties of phosvitin kinase from the estrogen-treated rooster liver. After purification by ammonium sulfate fractionation and chromatography on 10% agarose and cellulose phosphate, the purified enzyme appeared almost homogeneous and was recovered in a high yield. The single most effective step in purification was the cellulose phosphate chromatography, which presumably acted like a substrate affinity column with the phosphate groups of the cellulose phosphate interacting with the enzyme like the phosphate groups of phosvitin. A preliminary physical characterization of phosvitin kinase indicated that it has a sedimentation coefficient of about 7.6 S. Since the enzyme showed a tendency to aggregate on gel filtration, an accurate estimation of its Stokes (molecular) radius was not possible. From the density centrifugation data, the minimal molecular weight of the native enzyme is about 160,000.
The availability of a purified preparation of phosvitin kinase will facilitate a study of the biosynthesis of phosvitin. Using the enzyme as a probe, it may be possible to determine at what stage in protein synthesis phosphorylation of phosvitin takes place, that is, whether it occurs at some step prior to peptide release from the microsomes or whether it occurs after peptide release. It may also be possible to determine whether all the serine moieties in phosvitin are phosphorylated by the same enzyme. Moreover, the purified enzyme will provide a means for labeling authentic phosvitin with 32P and this radioactive phosvitin marker should aid in the identification of phosvitin synthesized de novo from labeled amino acid precursors during cell-free protein synthesis.

REFERENCES


Fig. 10. Km determination for phosvitin. Each phosphatase kinase reaction was incubated at 37°C for 10 min under standard conditions except for the indicated phosvitin concentration and contained either 2 μg of phosphatase kinase from control roosters (ΟΟΟ) or 1.2 μg of phosphatase kinase from roosters treated with 30 mg of 17β-estradiol (ΟΟΟΟΟΟΟΟ) as described in the legend to Table IV. Both enzyme preparations were purified by the identical procedures of ammonium sulfate fractionation and cellulose phosphate chromatography. The amount of 32P incorporated at zero time (1.4 pmoles) was subtracted from all values. In the calculation of a Km value in moles per liter for phosvitin, a molecular weight of 40,000 was assumed (7) and no correction was made for the fact that the phosvitin was only 50% dephosphorylated.

The purified phosphatase kinase exhibited several properties that indicated that it is distinct from the class of cyclic AMP-dependent kinases. First, the purified enzyme phosphorylated only the acidic protein substrates phosvitin and casein and did not phosphorylate the basic protein substrates histone and protamine. Although the substrate specificity of the cyclic AMP-dependent protein kinases is not completely clear, phosphorylation of histone by this type of kinase occurs at a significantly greater rate than does phosphorylation of either casein or phosvitin (10). Second, phosphorylation of a variety of substrates by the purified phosphatase kinase was not stimulated by cyclic AMP. Third, the divalent cation requirements of phosphatase kinase activity in which Mg2+ was stimulatory and Ca2+ and Mn2+ were inhibitory is different from that reported for certain cyclic AMP-dependent kinases in which all three divalent cations stimulated phosphorylation (29). Finally, the observation that phosvitin kinase can utilize GTP as well as ATP as a phosphate donor does not appear to have been described as a characteristic of the cyclic AMP-dependent protein kinases. Whether the physiological substrate for hepatic phosphatase kinase of the estrogen-treated rooster is ATP alone, GTP alone, or both nucleotides remains to be determined. The use of GTP as a phosphate donor in phosphorylation has been reported previously in only a few other phosphorylation systems, including the phosphorylation of phosvitin by the phosphatase kinase from cerebral cortex of the ox (14), the enzymatic phosphorylation of an uncharacterized phosphoprotein fraction from calf thymus nuclei (30), and the enzymatic phosphorylation of a protein band in the 40 S subunit of ribosomal proteins from rabbit reticuloocytes (12). Although estrogen administration to roosters results in a marked induction in the hepatic synthesis of phosvitin (2-6, 25, 26), the specific activity of phosvitin kinase from the livers of treated and untreated roosters was not significantly different. These data suggest that phosphorylation of phosvitin is not a rate-limiting event in the over-all process by which estrogen acts to stimulate the synthesis of yolk proteins.
Phosvitin Kinase from the Liver of the Rooster: PURIFICATION AND PARTIAL CHARACTERIZATION
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