Phosvitin Kinase from the Liver of the Rooster

PURIFICATION AND PARTIAL CHARACTERIZATION*

JOSEPH L. GOLDSTEIN† and MARY ANN HASTY

From the Division of Medical Genetics, Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas 75235

SUMMARY

Phosvitin kinase, the enzyme that phosphorylates the egg yolk protein phosvitin, has been purified more than 8000-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 $S_{20,	ext{w}}$ 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. Mg$^{2+}$ stimulated the rate of phosphorylation, while Co$^{2+}$ and Mn$^{2+}$ 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 $K_m$ for ATP (7.7 μM). These data suggest that phosphorylation of phosvitin by phosvitin kinase is probably not a rate-limiting event in the over-all 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

$[\gamma^{32}\text{P}]\text{ATP}$ (14.6 to 31 Ci per mmole) and $[\gamma^{32}\text{P}]\text{GTP}$ (14 Ci per mmole) were purchased from New England Nuclear Corp. 17-$\beta$-Estradiol, phosvitin, casein, calf thymus histone (type

* This research was supported by United States Public Health Service Grant GM 19258-01.
† Recipient of a Research Career Development Award 1-K4-GM-70, 277-01 from the United States Public Health Service.
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. Dextazylose phosphate P-11 (Whatman) was obtained from H. Reeve Angel, Inc. Ovalbumin, human y-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 benzoate or 17-β-estradiol dissolved in benzyl benzoate, 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°, 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% (v/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. Lipid was removed from the protein precipitate by successive washings with 2 ml of hot ethanol; 4 ml of chloroform ether-ethanol (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 precipitate. 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°, 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° for 10 min, and the precipitate was collected on a Millipore filter (HAWP 0.25 μm) 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-diphenyloxazole (PPO) and 0.008% (w/v) 1,4-bis[2-(5-phenyloxazolyl)] benzene (POP0) 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°.

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 benzoate, 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°. 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 per 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 mM 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 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (Buffer B). Fraction III—Ten milliliters of Buffer II were applied to a column (90 × 2.5 cm) packed with Bio-Gel 10% agarose A-0.5M (200 to 400 mesh) 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 (56.1 mg) was dialyzed against buffer containing 50 mM Tris-Cl, pH 7.4, 0.5 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (Buffer C) and then applied to a cellulose phosphate P-11 column (6 × 1.5 cm) equilibrated in Buffer C. Fractions of 2.5 ml each were eluted by 50 ml of Buffer C at a flow rate of 50 ml per hour, followed by a 70-ml linear gradient of NaCl (0.5 to 1.2), followed by 25 ml of Buffer C containing 1.2 mM NaCl. The tubes containing phosvitin kinase activity, eluting at 0.9 mM 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 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (Buffer D). Aliquots of Fractions I, II, and III were stored at −190°. Fraction IV was stored at 0–4°. 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 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM 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° in a Beckman SW 50.1 rotor with Spinco model 1.2465F ultracentrifuge. At the end of 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 M 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 phosvitin (7), it can be estimated that the maximal concentration of phosvitin in plasma resulting from the administration of pharmacological amounts of estradiol was about 360 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 X 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 X g supernatant (Fraction I). No phosvitin-dependent activity in significant excess of that due to endogenous 32P incorporation was detected in the 900 X g pellet (crude nuclei), the 26,000 X g pellet (mitochondrial fraction), or in the 100,000 X 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 6% 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-
Fig. 2 (left). Bio-Gel 10% agarose A-0.5M chromatography of phosvitin kinase. The details of chromatography are given under "Materials and Methods." Phosvitin kinase (Fraction II, 222 mg of protein) was eluted from the column with buffer containing 50 mM Tris-Cl, pH 7.4; 0.1 M NaCl; 5 mM 2-mercaptoethanol; and 0.1 mM EDTA. Fractions of 5 ml were collected and 5-µl aliquots were assayed for phosvitin kinase activity (■—■) under the standard conditions at 37° for 8 min. The amount of 32P incorporated into phosvitin without additions of column fractions (1.3 pmoles) was subtracted from all values. The protein concentration (O—O) of each fraction was measured by the method of Lowry et al. against a buffer blank.

Fig. 3 (center). Cellulose phosphate chromatography of phosvitin kinase. The details of chromatography are given under "Materials and Methods." Phosvitin kinase (Fraction III, 45.6 mg of protein) was eluted from cellulose phosphate by a linear NaCl gradient (70 ml, 0.5 to 1.2 M NaCl). Fractions of 2.5 ml were collected and aliquots of 25 µl were assayed for phosvitin kinase activity (■—■) under the standard conditions at 37° for 15 min. The amount of 32P incorporated into phosvitin without additions of column fractions (0.9 pmoles) was subtracted from all values. The protein concentration (O—O) of each fraction was measured by the method of Lowry et al. against a buffer blank.

Fig. 4 (right). Density gradient centrifugation of phosvitin kinase. Of 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 (O—O) 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 pmoles) was subtracted from all values.

**Table I**

**Purification of phosvitin kinase**

Phosvitin kinase was prepared as described under "Materials and Methods." At each major step in the purification, aliquots of Fractions I to IV 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) after dialysis against H2O. Specific activity is defined as the number of nanomoles of 32P incorporated from [γ-32P]ATP into phosvitin per min per mg of protein under the standard assay conditions. One unit equals 1 n mole of 32P incorporated per min.

| Fraction | Protein | Specific activity | Purification | Total activity | Recovery % initial activity |
|----------|---------|------------------|--------------|---------------|--------------------------|
|          | Applied | Recovered        |              |               |                          |
|          | mg      | mg               | units/mg     | -fold         | units                    |
| I. S-105 | 311     | 311              | 0.057        | 1.0           | 17.7                     | 100                       |
| II. Ammonium sulfate, 0 to 60% | 311 | 222 | 0.17 | 3.0 | 37.7 | 55.1 |
| III. 10% agarose | 222 | 56.1 | 0.39 | 6.8 | 21.9 | |
| IV. Cellulose phosphate | 45.6 | 0.04 | 466.2 | 8179 | 18.6 | 49.3 |

**vitin 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, chymotrypsinogen, 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 bromphenol 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 Mg++, 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 Mg++, Co++, and Mn++ on rates phosphorylation of phosvitin is shown in Fig. 8. Mg++ exerted a marked stimulatory effect in the pH range between 6 and 8. At pH 6.6, the Mg++ influence was maximal at concentrations between 7 and 20 mM (Fig. 8A), while at pH 7.4 the Mg++ effect was maximal at concentrations between 1.5 and 6 mM (Fig. 8B). Co” and Mn++ inhibited phosvitin kinase activity at both pH 6.6 (data not shown) and pH 7.4 (Fig. 8B).

The monovalent cations Na+ and K+ had no significant effect on phosvitin kinase activity in the concentration ranges that were tested (0 to 0.1 M).

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 [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.
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.

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 at either pH 6.6 (Fig. 9) or pH 7.4 (data not shown).}

Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of [32P]phosvitin phosphorylated by phosvitin kinase. The standard phosvitin kinase assay was scaled up 5-fold so that the reaction mixture contained in a final volume of 0.5 ml the following components: 50 mM potassium phosphate, pH 6.6; 7.5 mM MgCl2; 500 μg of phosvitin; 12.5 μg of phosvitin kinase; and 5 μM [γ-32P]ATP (600 cpm per pmole). After incubation at 37°C for 10 min, the reaction was stopped by addition of 2 ml of cold 10% trichloroacetic acid. The resulting trichloroacetic acid precipitate was suspended in 0.1 ml of solution containing 30 mM PIP, (pH 6.7), 1% sodium dodecyl sulfate, 10% glycerol, and 0.1% 2-mercaptoethanol, and the proteins were denatured and reduced by incubation at 100°C for 2 min. A 50-μl aliquot was then applied to a 15% gel containing 0.1% sodium dodecyl sulfate and subjected to electrophoresis at room temperature for 2 hr per tube using bromphenol blue as the tracking dye. After electrophoresis, the gel was crushed and fractions of 7 drops each were collected in separate counting vials using a Maizel Autogeldivider (Savant). Radioactivity of each fraction (●—●) was determined by liquid scintillation counting using as a scintillant for each vial 10 ml of solution containing 0.6 g/100 ml of 2,5-diphenyloxazole (PO), 5% (v/v) H2O, and 10% (v/v) Bio-Solv BBS-3 solubilizer (Beckman) in toluene. The position of migration of phosvitin was determined by electrophoresis in a parallel gel of 75 pg of authentic phosvitin, which was identified by staining with 5 mg/100 ml of toluidine blue (lop).

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.

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 reaction rate was about 10-fold faster with phosvitin than with casein may be explained in part by the fact that phosvitin contains about 10 times more serine residues than casein. Both substrates were about 50% dephosphorylated when added to the assay. Since both phosvitin and casein contain blocks of 4 or more serine molecules in a row (28), it is possible that this unique structural sequence is the recognition site for the phosvitin kinase enzyme. Protamine, calf thymus histone (tested in the presence and absence of 0.1 mM cyclic AMP), and bovine serum albumin were not phosphorylated by phosvitin kinase at either pH 6.6 (Fig. 9) or pH 7.4 (data not shown).

Fig. 8. Effect of divalent cation concentration on the activity of phosvitin kinase. A, at pH 6.6, each reaction was incubated at 37°C under the standard conditions and contained 0.8 μg of phosvitin kinase and the indicated Mg2+ concentration. B, at pH 7.4, each reaction was incubated at 37°C for 10 min under the standard conditions (except that 50 mM Tris-Cl, pH 7.4, was used in place of 50 mM potassium phosphate, pH 6.6) and contained 1.2 μg of phosvitin kinase and the indicated concentration of either Mg2+, Co2+, or Mn2+. The amount of 32P incorporated at zero time (1.5 pmole) was subtracted from all values.

| Addition         | Kinase activity | Percentage of control |
|------------------|-----------------|----------------------|
|                  | pmoles 32P incorp./10 min |                      |
| Experiment A     |                 |                      |
| Complete         | 42.3            | 100                  |
| + Adenosine      | 42.2            | 100                  |
| + AMP            | 40.8            | 96                   |
| + Cyclic AMP     | 41.3            | 98                   |
| + ADP            | 3.0             | 7.1                  |
| Experiment B     |                 |                      |
| Complete         | 43.5            | 100                  |
| + Guanosine      | 43.6            | 100                  |
| + GMP            | 40.0            | 92                   |
| + Cyclic GMP     | 47.1            | 108                  |
| + GDP            | 18.2            | 42                   |
| + GTP            | 10.1            | 23                   |
| + CMP            | 46.0            | 106                  |
| + CDP            | 43.8            | 101                  |
| + CTP            | 44.3            | 102                  |
| + UMP            | 42.6            | 98                   |
| + UDP            | 39.3            | 90                   |
| + UTP            | 41.3            | 95                   |
| Experiment C     |                 |                      |
| Complete         | 63.4            | 100                  |
| + ITF            | 26.5            | 42                   |
| + TDP            | 52.8            | 83                   |
| + TTP            | 53.6            | 85                   |

* 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 7.1 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 $[\gamma-^{32}P]ATP$ and $[\gamma-^{32}P]GTP$ as phosphate donors for phosvitin kinase

Each reaction was incubated at 37° for 15 min under the standard conditions except for the indicated variation in phosphate donor and contained 2 μg of phosvitin kinase. Both $[\gamma-^{32}P]ATP$ and $[\gamma-^{32}P]GTP$ were present at a final concentration of 6 μM. The amount of $^{32}P$ incorporated at zero time (1.6 pmoles) was subtracted from all values.

| Phosphate donor | Kinase activity (pmoles $^{32}P$ incorporated/15 min) |
|-----------------|--------------------------------------------------|
| $[\gamma-^{32}P]ATP$ |                                        |
| – Phosvitin      | 0.8                                               |
| + Phosvitin      | 159.5                                             |
| $[\gamma-^{32}P]GTP$ |                                       |
| – Phosvitin      | 0.7                                               |
| + Phosvitin      | 86.5                                              |

### 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 17β-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 47 to 89 μg of a 0 to 60% ammonium sulfate fraction of liver supernatant prepared as described under "Materials and Methods." One unit of phosvitin kinase activity equals 1 n mole of $^{32}P$ incorporated per min.

| Animal          | Liver weight  | Plasma phosphoprotein | $^{32}P$ Incorporated into phosvitin |
|-----------------|---------------|-----------------------|-------------------------------------|
|                 | g             | μg/ml                 | Specific activity | Total activity |
|                 |               |                       | units/mg soluble protein | units/liver   |
| Control         |               |                       |                        |               |
| 1               | 34.8          | 1.2                   | 0.065                  | 2262          |
| 2               | 34.7          | 1.0                   | 0.092                  | 3192          |
| 3               | 21.6          | 1.2                   | 0.083                  | 1735          |
| Estradiol-treated |             |                       |                        |               |
| 4               | 80.7          | 101.9                 | 0.056                  | 4519          |
| 5               | 83.7          | 167.9                 | 0.077                  | 6444          |
| 6               | 60.1          | 88.2                  | 0.070                  | 4207          |

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 date, the minimal molecular weight of the native enzyme is about 160,000.
The specific activity of phosvitin kinase from the livers of treated and untreated roosters was not significantly different. Although estrogen administration to roosters results in a

rate-limiting event in the over-all process by which estrogen acts to stimulate the synthesis of yolk proteins. 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

1. Heald, P. J., and McLachlan, P. M. (1963) Biochem. J. 87, 571-576
2. Heald, P. J., and McLachlan, P. M. (1965) Biochem. J. 94, 32-33
3. Scherrer, O. A., and Under, M. B. (1966) Science 154, 1424-1445
4. Greenberg, O., Gordon, M., Smith, M. A., and Ac, G. (1965) J. Biol. Chem. 239, 2079-2082
5. Greenberg, O., Gentzak, A., and Aus, G. (1965) J. Biol. Chem. 240, 1687-1991
6. Maenpaa, P. R., and Bernfeld, M. B. (1969) Biochemistry 8, 4926-4933
7. Allerton, S. E., and Perlmann, G. E. (1965) J. Biol. Chem. 240, 3392-3398
8. Burnett, G., and Kennedy, E. P. (1954) J. Biol. Chem. 219, 891-900
9. Sundarajanan, T. A., Kumar, K. S. V., and Sarna, P. S. (1958) Biochim. Biophys. Acta 29, 449-450
10. Blankenstutz, M., and Lipman, F. (1966) J. Biol. Chem. 231, 1042-1050
11. Puc, G. A., Nola, E., Sica, V., and Brseciati, F. (1972) Biochim. Biophys. Res. Commun. 49, 970-976
12. Truog, J. A., Mucdroy, M., and Traut, R. R. (1973) Proc. Nat. Acad. Sci. U. S. A. 70, 373-376
13. Judah, J. D., Ahmed, K., and McLean, A. E. M. (1962) Biochim. Biophys. Acta 64, 412-418
14. Rodnight, R., and Layin, B. E. (1964) Biochem. J. 93, 84-91
15. Krane, S. M., Stone, M. J., and Glimescher, M. J. (1965) Biochim. Biophys. Acta 97, 77-87
16. Jackson, J. E., Jackson, E. M., and Freemman, S. (1965) Biochim. Biophys. Acta 105, 483-485
17. Wallace, R. A. (1964) Biochem. Biophys. Acta 66, 286-294
18. Masso, Y., and Lipman, F. (1962) J. Biol. Chem. 241, 3834-3837
19. Krebs, E. G. (1972) Curr. Top. Cell. Regul. 5, 99-123
20. Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 65, 375-378
21. Lowry, O. H., Rosebrooxh, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
22. Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1572-1579
23. Maizel, J. V. (1971) in Methods in Virology (Maramorosch, K., and Koprowski, H., eds) Vol. 5, pp. 179-246, Academic Press, New York
24. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
25. Jaiklewe, B. L., and Talwar, G. P. (1972) Nature New Biol. 235, 236-240
26. Retiwing, G., and Gutter, M. (1971) Biochim. Biophys. Acta 292, 524-528
27. Smith, M. H. (1970) in Handbook of Biochemistry, Selected Data for Molecular Biology (Chen, H. A., ed) 2nd Ed, C-3, The Chemical Rubber Co., Cleveland
28. Williams, J., and Sanger, F. (1956) Biochim. Biophys. Acta 33, 294-296
29. Majumder, G. C., and Torkington, R. W. (1971) J. Biol. Chem. 246, 2910-2917
30. Kleinman, L. J., and Allfrey, V. G. (1969) Biochim. Biophys. Acta 175, 123-135
31. Kleinman, L. J., and Allfrey, V. G. (1969) Biochim. Biophys. Acta 175, 123-135

Fig. 10. Km determination for phosvitin. Each phosvitin kinase reaction was incubated at 37° for 10 min under standard conditions except for the indicated phosvitin concentration and contained either 2 µg of phosvitin kinase from control roosters (O) or 1.5 µg of phosvitin kinase from roosters treated with 30 mg of 17β-estradiol (●—●) 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 the 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.
Phosvitin Kinase from the Liver of the Rooster: PURIFICATION AND PARTIAL CHARACTERIZATION
Joseph L. Goldstein and Mary Ann Hasty

J. Biol. Chem. 1973, 248:6300-6307.

Access the most updated version of this article at http://www.jbc.org/content/248/18/6300

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/248/18/6300.full.html#ref-list-1