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Polypeptide-dependent protein kinase from bakers' yeast

(histone/H$^+$-ATPase/membrane protein kinase/RNA-cap-binding protein/GTP-binding protein)

Y. YANAGITA*, M. ABDEL-GHANY, D. RADEN, N. NELSON†, AND E. RACKER‡

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853

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ABSTRACT **The purification and properties of a protein serine kinase (PK-P) extracted with Triton X-100 from membranes of bakers' yeast are described. The enzyme is virtually inactive unless either a histone or a heat-stable polypeptide from yeast membranes and Mg^{2+} are added. Other divalent cations substitute for Mg^{2+} poorly or not at all; most of them, including Mn^{2+}, inhibit when added in the presence of 5 mM Mg^{2+}. The enzyme is unstable but can be stabilized by addition of 0.1% Triton X-100 and 20% glycerol. The final preparation shows, on silver-stained electrophoresis gels, two major bands (M_r 41,000 and 35,000). According to gel filtration the molecular weight of the active protein is about 75,000. Of the two subunits, only the smaller one appears to be autophosphorylated. In addition to casein, the enzyme phosphorylates several proteins including the H$^+$-ATPase (M_r 100,000) in the yeast plasma membrane. In order to demonstrate the phosphorylation of the ATPase (up to 0.9 equivalents), exposure of the latter to an acid phosphatase was required. Other phosphorylated proteins include mRNA cap-binding protein from mammalian erythrocytes and yeast, a glucocorticoid receptor protein, and a preparation of the guanine nucleotide-binding proteins G_i and G_o from brain. A partial purification of a natural activator from yeast plasma membranes is described.**

Protein phosphorylation and dephosphorylation have been widely recognized as regulatory mechanisms of metabolism, membrane function, and structural as well as contractile proteins (1). The physiological role of many, if not most, protein kinases that have been described has remained unknown, particularly in the case of membranous protein kinases that phosphorylate either serine, threonine, or tyrosine residues in proteins. In view of the rapid advances in molecular genetics, it seems likely that new clues for the physiological function of protein kinase will be obtained from studies of yeast protein kinases. The gene for one protein kinase that is required for release from glucose repression has recently been cloned. The gene product, a M_r 72,000 protein, was shown to be autophosphorylated (2). The gene for another protein kinase is *CDC28*, which is required for initiation of the cell cycle. The molecular weight of this enzyme is 36,000 and its activity is dependent on Zn^{2+} (3).

Previous reports from this laboratory described the partial purification of a cAMP-independent protein kinase from plasma membranes of placenta (4) and Ehrlich ascites tumors (5). The enzyme required a heat-stable polypeptide for activity, which was isolated from placenta and identified as histone 1 (6). It was shown that this membranous polypeptide-dependent protein kinase is distinct from other protein kinases. We describe in this paper the purification and characterization of a similar enzyme (PK-P) from membranes of bakers' yeast. This enzyme was activated by polypeptides with a high content of lysine, such as histone 1, or by an endogenous polypeptide which was partially purified from bakers' yeast membranes.

MATERIALS AND METHODS

Materials. Aquacide III (polyethylene glycol, M_r 20,000) was a product of Calbiochem. Triton X-100 (membrane research grade) was obtained from Boehringer Mannheim. Thioglycerol was from Evans Chemetics (Waterloo, NY). Casein (Sigma no. C 4765) was heated at 10 mg/ml for 5 min at 80°C to remove residual protein kinase activity. Amylopectin sulfate (with 14.6% S) was a gift from P. Cammarata (Searle, Chicago). Pressed bakers' yeast (Red Star brand) was used as a source of PK-P. [γ-^{32}P]ATP was purchased from Amersham; DE-23 DEAE-cellulose, from Whatman; Bio-Gel HTP, from Bio-Rad; and Sephacryl S-300 superfine, from Pharmacia. Poly(Ser^{25}Lys^{75}) random copolymer was a gift from E. Katchalski (Weizmann Institute, Israel). Two hundred milligrams of this polymer was coupled to 5 g of aminohexyl-Sepharose 4B (Pharmacia) according to the method described in the Pharmacia manual, and the coupled Sepharose is referred to as poly(Ser^{25}Lys^{75})-Sepharose. Histone 1 (type IIIS, catalog no. H 5505) was purchased from Sigma. Fifty-three milligrams of histone 1 was coupled to 2.5 g of CNBr-activated Sepharose 4B (Pharmacia), and the coupled Sepharose is referred to as histone-Sepharose. Heparin-agarose (H 6508) and acid phosphatase (P 6760) were purchased from Sigma. Altex Spherogel TSK phenyl 5PW was purchased from Beckman.

Buffers. Buffer A contained 20 mM Na Hepes (pH 7.4), 10 mM thioglycerol, and 10% (vol/vol) glycerol. Buffer A/TX contained 0.1% (vol/vol) Triton X-100 in buffer A. Buffer B contained 20 mM Na Hepes and 100 mM sodium phosphate (pH 6.7).

Assay of PK-P. The assay mixture (50 μl) contained 20 mM Na Hepes (pH 7.4), 5 mM $MgCl_2$, 10 mM thioglycerol, 5 μg of heated casein with or without 5 μg of histone 1, 10 μM [γ-^{32}P]ATP (3000–4000 cpm/pmol), and 5 μl of enzyme (1 μg to 0.5 ng) protein diluted with buffer A/TX. Amylopectin sulfate (0.5 μg; potato amylopectin sulfate, $M_r \approx 36 \times 10^6$, 14.76% S) was added to the assay mixture when PK-P activity in the crude extract was measured. The reaction was started by adding radioactive ATP. After 30 min at room temperature, 20 μl was placed on Whatman 3 MM filter paper and analyzed as described (5). Phosphorylations without histone were subtracted from the values with histone to calculate polypeptide-dependent protein kinase activity. One unit of PK-P was defined as 1 nmol of activator-dependent phosphorylation of casein per min.

Assay of a Natural Activator of PK-P. PK-P (0.45 ng) at a specific activity of about 700 nmol per min per mg of protein was tested in the assay medium described above in the

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*Present address: Jichi Medical School, Minamikawachi-Machi, Kawachi-Gun, Tochigi-Ken, Japan 329-04.
†Present address: Department of Biochemistry, Roche Institute of Molecular Biology, Nutley, NJ 07110.
‡To whom reprint requests should be addressed.

presence of 0.2–1 μg (protein) of the natural activator. With each assay a standard curve with histone at the same range of proteins was established. Specific activity was expressed as the ratio of activation compared to that obtained with the same amount of histone.

Dephosphorylation and Rephosphorylation of the H^+-ATPase of Yeast Plasma Membranes. In a final volume of 60 μl, yeast ATPase (0.1 mg/ml) was incubated with acid phosphatase (0.5–2.5 μg/ml) in 2.5 mM sodium acetate, pH 5.0/2.5 mM $MgCl_2$ at 30°C. After specific times, 60 μl of 100 mM Tris Cl (pH 8.3) was added and the mixture was incubated for 30 min at 30°C to inactivate the phosphatase. The enzyme was then rephosphorylated with 1.8 ng of purified PK-P as described above with [γ-^{32}P]ATP (3900 cpm/pmol). Calculations of stoichiometry were performed assuming a molecular weight of 100,000 for ATPase.

Assay of Yeast Plasma Membrane ATPase. The assay mixture contained, in a final volume of 200 μl, 25 mM Na Mes (pH 6.0), 50 mM NaCl, 5 mM $MgCl_2$, and 5 mM Na ATP. The ATPase reaction was started by adding ATPase (0.2–1 μg). After 15 min at 30°C, the reaction was terminated by adding 150 μl of 10% (wt/vol) $NaDodSO_4$, followed by 1.3 ml of ammonium molybdate solution (5.6 mg of ammonium molybdate per ml of 1.39 M H_2SO_4) and 70 μl of ANS solution (2.5 mg of 1-amino-2-naphthol-4-sulfonic acid, 5 mg of Na_2SO_3, and 140.5 mg of $Na_2S_2O_5$ per ml). The mixture was incubated for 10 min at 37°C and measured for optical absorbance at 740 nm.

Isolation of Yeast Membranes. Two pounds of pressed bakers' yeast (Red Star) was crumbled with hands and frozen in 2–3 liters of liquid nitrogen. After the yeast was completely frozen, it was transferred into a stainless steel blender (Waring). The frozen pellets were homogenized at top speed for 3 min in three equal 1-min bursts. Between each burst, liquid nitrogen was supplied and the powder was redistributed inside the blender with a metal spatula. The final product was very fine dry powder. The powder was transferred to 1.5 liters of 0.4 M mannitol/0.05 M Tris Cl, pH 8.2/1 mM EDTA and was kept at room temperature. After all the powder was transferred and the suspension was completely thawed, the pH of the suspension was adjusted to 7.4–7.5 with 4 M NaOH. The suspension was transferred to the blender and homogenized for 1 min at top speed.

The homogenate was centrifuged in a Sorvall GS3 rotor for 20 min at 3000 rpm (1520 × *g* max). The supernatant was collected through four layers of cheesecloth and centrifuged in a GS3 rotor for 45 min at 8000 rpm (10,800 × *g* max). The supernatant was collected as described above and centrifuged for 30 min at 48,000 rpm in a Beckman Ti 60 ultracentrifuge rotor. The pellet was collected and homogenized with 200 ml of 0.25 M mannitol/20 mM Tris Cl, pH 7.5, using a Potter homogenizer. The suspension was centrifuged as described above, and the pellet was resuspended in ≈100 ml of the same buffer. (All centrifugation steps were done at 0–4°C.) The suspension was frozen in liquid nitrogen and stored at −70°C. Two pounds of yeast usually yielded 5–7 g of membrane proteins.

Solubilization of PK-P. The membrane suspension containing 5 g of protein (70–100 ml) was thawed and mixed with 500 ml of 2× buffer A (4°C) and 100 mg of soybean trypsin inhibitor (Sigma T 9003). The volume of the suspension was adjusted to 975 ml with cold water. Then 25 ml of 20% (vol/vol) Triton X-100 was added and the whole mixture was incubated for 20 min at 4°C with constant stirring. After incubation, the mixture was centrifuged at 4°C for 60 min at 48,000 rpm. The resulting supernatant (≈950 ml, 2–2.5 mg of protein per ml) was used as the starting material for purification.

Purification of PK-P. All steps were done at 0–4°C unless stated otherwise. *Step 1. DE-23 chromatography.* The solubilized membranes (≈950 ml) were loaded onto a Whatman DE-23 anion-exchange cellulose column (5.9 × 18.3 cm) equilibrated with buffer A/TX. The column was washed first with 500 ml of buffer A/TX, then with 500 ml of buffer A/TX containing 0.15 M NaCl. PK-P was eluted with a linear gradient of NaCl (0.15–0.65 M, 1250 ml each) in buffer A/TX. Fractions of 25 ml were collected, and the fractions with highest specific activity were combined.

Step 2. Bio-Gel HTP chromatography. The combined fractions from step 1 (400–500 ml) were loaded on a Bio-Gel HTP column (5.9 × 3.6 cm) equilibrated with buffer A/TX. The column was washed first with 100 ml of buffer A/TX, then with 100 ml of buffer A/TX containing 0.1 M potassium phosphate (pH 7.4). PK-P was eluted with a linear gradient of potassium phosphate (0.1–0.5 M, 200 ml each) in buffer A/TX. Fractions of 10 ml were collected. The fractions with the highest specific activity were pooled and dialyzed overnight against 2 liters of buffer A/TX, in which 300 g of Aquacide III had been dissolved per liter of liquid.

Step 3. Sephacryl S-300 chromatography. The concentrated fraction of step 2 (≈50 ml) was loaded on a Sephacryl S-300 column (7 × 42 cm) equilibrated with buffer A/TX containing 0.1 M NaCl. PK-P was eluted from the column with buffer A/TX/0.1 M NaCl at a flow rate of 75 ml/hr, and fractions of 25 ml were collected. The fractions with the highest specific activity were combined and dialyzed overnight against 2 liters of buffer A/TX/0.1 M NaCl, in which 300 grams of Aquacide III had been dissolved per liter of liquid.

Step 4. Poly(Ser^{25}Lys^{75})-Sepharose chromatography. Two batches of the concentrated eluate from step 3 (≈80 ml each) were loaded on a poly(Ser^{25}Lys^{75})-Sepharose column (1.25 × 14.5 cm) equilibrated with buffer A/TX/0.1 M NaCl. The column was washed first with 20 ml of buffer A/TX/0.1 M NaCl, then with 20 ml of buffer A/TX/0.5 M NaCl. PK-P was eluted with a linear gradient of NaCl (0.5–1.5 M, 50 ml each) in buffer A/TX. Fractions of 2.4 ml were collected. The fractions with highest specific activity were pooled and dialyzed overnight against 2 liters of buffer A/TX.

Step 5. Heparin-agarose chromatography. Three batches of the dialyzed eluate from step 4 (≈40 ml each) were loaded on a heparin-agarose column (1.0 × 12.0 cm) equilibrated with buffer A/TX. The column was washed with 20 ml of buffer A containing 0.1 M potassium phosphate (pH 7.4). PK-P was eluted from the column with a linear gradient of potassium phosphate (0.1–0.7 M, 30 ml each) in buffer A. Fractions of 1.5 ml were collected. The fractions with highest specific activity were pooled and dialyzed overnight against 1 liter of buffer A.

Step 6. Histone-Sepharose chromatography. The dialyzed eluate from step 5 (≈15 ml) was mixed with 0.5 ml (wet volume) of histone-Sepharose (equilibrated with buffer A) and incubated for 3.5 hr at 4°C in an end-over-end shaker. After incubation, the suspension was poured into a 2.5-ml disposable plastic syringe with a plastic filter at the bottom. The column was washed with 1.5 ml of buffer A. PK-P was eluted with 1.5 ml of 0.3 M NaCl in buffer A and kept frozen at −70°C.

Step 7. Phenyl 5PW HPLC. This step was done at room temperature. A column of TSK phenyl 5PW (7.5 × 75 mm) was equilibrated with buffer B containing 0.9 M $(NH_4)_2SO_4$ and set at a flow rate of 0.75 ml/min. The eluate from step 6 (1.5 ml) was mixed with 0.5 ml of saturated $(NH_4)_2SO_4$ (pH 7.4) and injected into the column (two times, 1 ml each). The column was washed with the starting buffer for the first 6 min after the injection of the sample. Then the adsorbed proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ (0.9–0.0 M, 8 ml each) in buffer B, followed by buffer B. Fractions of 0.75 ml were collected into tubes to which 0.2 ml of 50% (vol/vol) glycerol had been added. As shown in Fig. 1, the activity emerged in a single peak. The indicated three fractions

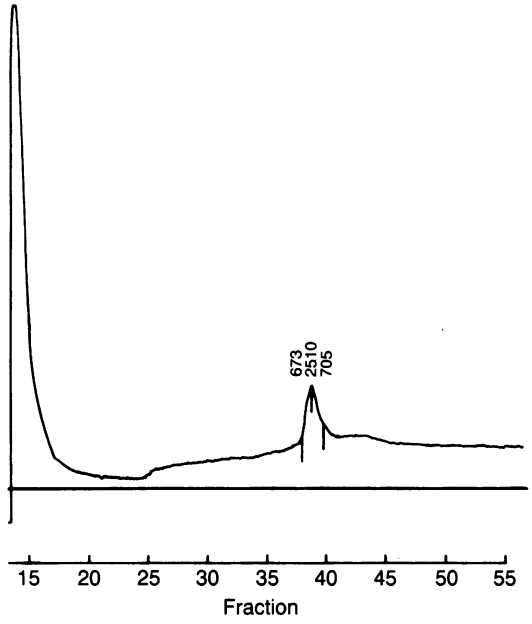

FIG. 1. Purification of PK-P by TSK phenyl 5PW HPLC. The ordinate represents absorbance at 250 nm. The three tubes (fractions 38–40) with the indicated activities (units/ml) were pooled for further analysis.

containing the majority of the enzyme were pooled and Triton X-100 was added to a final concentration of 0.1% (vol/vol). The mixture was then divided into aliquots, quickly frozen in liquid nitrogen, and kept at −70°C.

Other Methods. Proteins were measured as described by Bradford (7), with bovine serum albumin as a standard. $NaDodSO_4$/polyacrylamide gel electrophoresis was done with 12% (wt/vol) acrylamide as described by Laemmli (8). The thickness of the gel was 0.75 mm. Autoradiography was performed using Kodak XAR-5 x-ray film with photointensifying screens and exposed at −70°C for 1–4 days.

RESULTS

Purification of Yeast PK-P. Yeast PK-P was purified about 6000-fold, starting with a Triton X-100 extract of yeast membranes (Table 1). The assay of PK-P in crude extracts is not accurate because of the presence of other protein kinases that phosphorylate casein. Since yeast PK-P was much less sensitive to inhibition by amylopectin sulfate (or other acidic polysaccharides) than other protein kinases present in the crude extract of plasma membranes, addition of this inhibitor during assay allowed the determination of histone 1-dependent activity. The values thus obtained may still represent an overestimate, which would account for the apparent poor recovery of activity from the DE-23 column. Alternatively, the enzyme in the crude extract may be in an activated form due to the presence of endogenous activator. The presence of an activator of PK-P in the plasma membrane will be shown below. After elution from DE-23, the assay was performed in the absence of amylopectin sulfate, since a marked stimulation of activity by histone 1 was observed and the total activity was only slightly affected by the inhibitor. The specific activity of the final PK-P preparations was about 1 μmol per min per mg of protein. $NaDodSO_4$/PAGE revealed two major bands (M_r 41,000 and 35,000) by silver staining (Fig. 2*A*). The diffuse bands at about M_r 66,000 are silver-stain artifacts that were absent without mercaptoethanol but were seen in lanes that contained mercaptoethanol but no protein. Autoradiograms of this preparation revealed the autophosphorylation of the M_r 35,000 band but not of the M_r 41,000 band as well as a stimulation by histone 1 (Fig. 2*B*).

Properties of Yeast PK-P. The success in the purification of yeast PK-P was predicated on the presence of both Triton X-100 and glycerol, which were required for the preservation of enzyme activity. The enzyme exhibited a pH optimum between 7 and 9 with a sharp drop in activity at pH 6 and 10 (Fig. 3*A*). An optimal Mg^{2+} concentration between 2 mM and 10 mM was required, and inhibition by Mg^{2+} was observed above 20 mM (Fig. 3*B*). Other divalent cations substituted poorly. Mn^{2+}, which was the best, gave rise to about 30% of maximal activity at an optimal concentration of 0.1 mM. Even at this concentration the activity in the presence of 5 mM Mg^{2+} was sharply curtailed. Other cations inhibited also, including Ca^{2+}, which in the absence of Mg^{2+} did not stimulate. Phospho amino acid analysis of casein phosphorylated by PK-P, performed as described (9), revealed that 90% of the radioactivity was present in serine residues and 10% in threonine residues.

During assay, high salt concentration must be avoided, since KCl or NaCl inhibited at >50 mM, whereas the potassium or sodium salts of either sulfate or phosphate inhibited at concentrations above 5 mM, with phosphate being tolerated better than sulfate. NaF inhibited strongly at 10 mM. Quercetin inhibited 50% at 0.55 μg/ml, and vanadate at 2.2 mM. At 0.1 mM no inhibition by vanadate, which inhibits H^+-ATPase of plasma membranes, was observed.

Since casein is obviously not a natural substrate for the yeast enzyme, the plasma membrane of yeast was examined for the presence of endogenous substrates that were more extensively phosphorylated by radioactive ATP in the presence of histone 1. Of particular interest was the finding that purified preparations of the yeast H^+-ATPase were phosphorylated by added PK-P in the presence of histone 1 (Fig. 4*A*). A pure preparation of H^+-ATPase from the plasma mem-

Table 1. Purification of PK-P

Step	Volume, ml	Activity*		Protein		Specific activity†	Purification, fold	Yield, %
		Units	Units/ml	mg	mg/ml			
Solubilized membranes	5587	2386	0.3	12,632	2.26	0.19	1	100
DE-23	2375	672	0.28	1,536	0.647	0.44	2.3	28
Bio-Gel HTP	2008	595	0.30	759	0.378	0.8	4.2	25
Sephacryl S-300	1050	496	0.47	102	0.097	4.9	26	21
Poly($Ser^{25}Lys^{75}$)-Sepharose	122	304	2.49	17.9	0.147	17.0	90	13
Heparin-agarose	15	90	6.05	2.23	0.149	40.7	215	3.8
Histone-Sepharose	1.5	49	33.2	0.48	0.320	103.8	549	2.1
TSK phenyl 5PW	2.7	28	10.4	0.025	0.009	1114.0	5894	1.2

Protein was determined according to Bradford (7), except for the last step, where the amount of protein was estimated by silver staining of the gel using various concentrations of bovine serum albumin as standards.
*One unit = 1 nmol of P_i incorporated per min.
†Nanomoles of P_i per min per mg of protein.

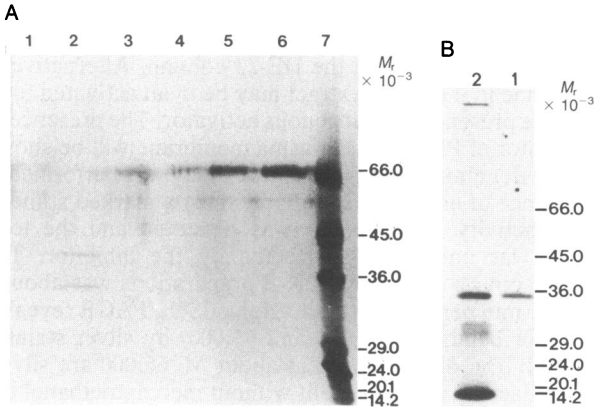

FIG. 2. (*A*) NaDodSO$_4$/PAGE analysis of purified PK-P by silver staining. Lanes 1 and 2: 10 and 25 μl of the purified enzyme of step 7. Lanes 3–6: 50, 100, 250, and 500 ng of bovine serum albumin. Lane 7: molecular weight standards. (*B*) Autophosphorylation of PK-P. The reaction mixture (50 μl) contained 20 mM Na Hepes (pH 7.4), 10 mM thioglycerol, 10% (vol/vol) glycerol, 5 μl of purified PK-P (step 7), 10 μM [γ-^{32}P]ATP (3900 cpm/pmol) in the absence or presence of 5 μg of histone 1. The mixture was incubated at room temperature for 30 min. The reaction was stopped by the addition of 25 μl of NaDodSO$_4$ sample buffer. The mixture was loaded on a NaDodSO$_4$/10% polyacrylamide gel. After electrophoresis for 5 hr at 30 mA, the gel was dried and exposed for 2 days to Kodak XAR-5 film. Lanes 1 and 2: PK-P in the absence and presence of histone 1, respectively.

brane of *Neurospora crassa* responded similarly. As shown in Fig. 4*B*, treatment of the enzyme with acid phosphatase resulted in a much greater extent of ^{32}P incorporation. Quantitative measurements of ^{32}P incorporation into the enzyme showed that after 60 min of treatment with 2.5 μg of phosphatase, the mole equivalence was 0.96 in the presence of histone 1 and 0.14 in its absence. Phosphorylation of several other proteins, including the RNA-cap-binding protein, the progesterone receptor, and some GTP-binding proteins, was also seen (data not shown), but the physiological meanings of these observations have not been established.

A Natural Activator of Yeast PK-P. A search for a natural activator in the plasma membrane revealed two peaks of activity emerging from a DE-23 column, one before and one after the emergence of PK-P on salt elution. The second peak was selected for further purification and study. The activator is nondialyzable, stable to 10 min of heating at 100°C, but sensitive to digestion by trypsin (Table 2).

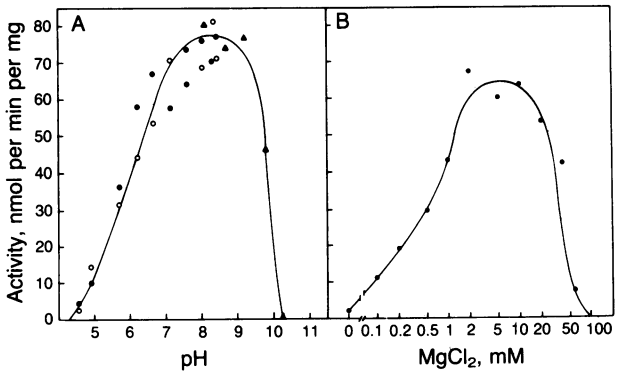

FIG. 3. pH optimum (*A*) and Mg^{2+} requirement (*B*) of PK-P. (*A*) Reaction mixtures contained 20 mM Tris Mes (pH 4.55–8.44) or 20 mM Na Caps (pH 8.11–10.30) instead of 20 mM Na Hepes. The enzyme was diluted first 5-fold with buffer A and then diluted further 10-fold with different dilution buffers (20 mM Tris Mes or Na Caps in place of Na Hepes in buffer A). Five microliters of the enzyme at various pH was analyzed in the reaction mixture at the same pH as used for dilution. Circles, Tris Mes; triangles, Na Caps. (*B*) Reaction mixtures contained various concentrations of $MgCl_2$ instead of the standard 5 mM. PK-P was diluted 50-fold with buffer A, and 5 μl of the diluted enzyme was analyzed.

DISCUSSION

PK-P from yeast is similar to the polypeptide-dependent protein kinase (PPdPK) from placenta and tumor cells (4–6).

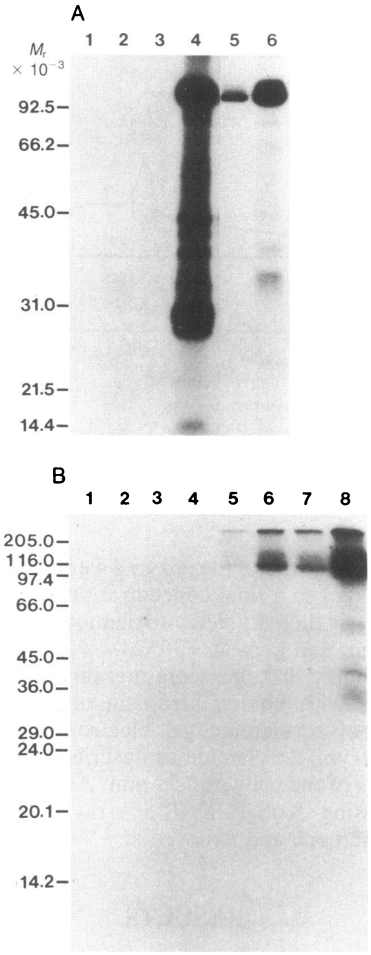

FIG. 4. (*A*) H$^+$-ATPase as a substrate for PK-P. Reaction mixtures (20 μl) contained 20 mM Na Hepes, 10 mM thioglycerol, 5 mM $MgCl_2$, 2 μl of purified PK-P (step 7) with or without 1 μg of histone 1, 10 μM [γ-^{32}P]ATP (4200 cpm/pmol), and 600 ng of plasma membrane H$^+$-ATPase from *Saccharomyces cerevisiae* or *Neurospora crassa*. Mixtures were incubated for 30 min at room temperature, and reactions were terminated by the addition of 15 μl of 4× dissociation buffer for NaDodSO$_4$/PAGE, followed by the addition of 25 μl of water. The whole mixture (60 μl) was loaded on a NaDodSO$_4$/polyacrylamide gel. Electrophoresis was done at constant current (4 mA) overnight. The gel was stained with silver and then subjected to autoradiography for 4 days. Lanes: 1, PK-P; 2, PK-P plus histone 1; 3, PK-P plus yeast H$^+$-ATPase; 4, PK-P plus yeast H$^+$-ATPase plus histone 1; 5, PK-P plus *N. crassa* H$^+$-ATPase; 6, PK-P plus *N. crassa* H$^+$-ATPase plus histone 1. (*B*) Dephosphorylation of yeast plasma membrane H$^+$-ATPase. Five microliters of ATPase (0.2 mg/ml) were mixed with 5 μl of acid phosphatase (0.5 μg/ml) in 50 mM Tris Mes, pH 5.0/5 mM $MgCl_2$ and incubated for 30 min at 37°C. Fifteen microliters of 100 mM Tris Cl (pH 8.3) was added to the mixture, and the mixture was kept for a further 30 min at 37°C. The ATPase was phosphorylated by 1.8 ng of PK-P. Thirty microliters was loaded on a gel. The gel was exposed to a Kodak XAR-5 film for 24 hr. Lanes: 1, yeast H$^+$-ATPase; 2, yeast H$^+$-ATPase plus histone 1; 3, yeast H$^+$-ATPase plus phosphatase; 4, yeast H$^+$-ATPase plus phosphatase plus histone 1; 5, yeast H$^+$-ATPase plus PK-P; 6, yeast H$^+$-ATPase plus PK-P plus histone 1; 7, yeast H$^+$-ATPase plus phosphatase plus PK-P; 8, yeast H$^+$-ATPase plus phosphatase plus PK-P plus histone 1.

Table 2. Heat stability and trypsin sensitivity of PK-P activator

Treatment	cpm
None (control)	3146
Trypsin (2 μg, 37°C, 30 min)	214
Trypsin inhibitor (20 μg, 37°C, 30 min)	3212
Trypsin inhibitor + trypsin (37°C, 30 min)	2824
Heat (95°C, 7 min)	3248

In a final volume of 15 μl, 5 μg of the activator was incubated with 2 μg trypsin at 37°C for 30 min. Proteolysis was terminated by addition of 5 μl (20 μg) of soybean trypsin inhibitor. In a control experiment, trypsin was mixed with trypsin inhibitor prior to the addition to activator. Trypsin inhibitor alone had no effect. For the determination of heat stability the activator was incubated at 95°C for 7 min at a protein concentration of 0.2 mg/ml. Activation was assayed as described under *Materials and Methods*.

In view of previous publications on casein kinases from yeast, it is essential to show that PK-P is different from those described. One of the best characterized yeast protein kinases was described by Lerch *et al.* (10). Like PK-P, it phosphorylates casein but not histones and has a similar pH optimum. However, the enzyme was isolated from the cytosol and did not require Triton X-100 for stability. Moreover, we observed no stimulation on addition of histone 1 to a highly purified preparation of this enzyme and no inhibition by amylopectin sulfate at high concentrations that inhibit both casein kinase II and PK-P (data not shown). Another protein kinase from yeast (11, 12) greatly resembles casein kinase II from mammalian sources. This enzyme was present in crude preparations of PK-P. It is a soluble enzyme and was inhibited about 80% by heparin (0.8 μg/ml) or other acidic polysaccharides, such as amylopectin sulfate, whereas PK-P was not. Differences between casein kinase II and polypeptide-dependent protein kinase from tumor cells were discussed in a previous report (6). They are also seen with yeast PK-P. Recently described properties of casein kinase II from *Saccharomyces cerevisiae* (11, 12) are clearly different from those of PK-P. Casein kinase II is described as a homotetramer of M_r 150,000, consisting of four autophosphorylated subunits of M_r 37,000, whereas we propose that PK-P is a heterodimer of M_r ≈75,000, with only one phosphorylated subunit. We have not ruled out the possibility that PK-P is a single polypeptide that has been nicked by an endogenous protease. Stimulation of casein kinase II by polyamines was shown to be at best 3-fold, and autophosphorylation was inhibited by polylysine. PK-P shows an almost complete dependence on polylysine-rich polypeptides, which also stimulate autophosphorylation. The substrate specificity appears to differ also since UDP glycogen synthase was reported to be a substrate for casein kinase II, whereas PK-P did not phosphorylate highly purified UDP glycogen synthase (kindly provided by P. J. Roach, Indiana University, Indianapolis).

As discussed previously (6), basic activators of casein kinase II from various sources have been described. The question has been raised whether the enzyme or the casein substrate is activated (13). The observation that the autophosphorylation of the enzyme is stimulated by histone 1 does not settle the question but clearly demonstrates an interaction of the histone with the enzyme. Recently, we have observed the presence of a heat-stable, nondialyzable component in yeast that inhibited PK-P but not casein kinase II.

Of particular interest is the question of the physiological role of PK-P. As shown in this paper, the H^+-ATPase of the plasma membrane is a natural substrate for the enzyme, but detailed studies have shown that <10% of the purified enzyme was phosphorylated in the presence of ^{32}P-labeled ATP. Among several alternatives to explain this finding, the most interesting possibility was that the isolated enzyme is already phosphorylated at most of its available sites. This was borne out by experiments described in this paper. RNA-cap-binding protein (14) from erythrocytes and from yeast is a substrate for PK-P. No other protein kinase has been observed to phosphorylate this protein, which is isolated as a phosphoprotein. Of particular interest are observations that the guanine nucleotide-binding proteins G_i/G_o from brain (M.A.-G. and R. Cerione, unpublished data) and the progesterone receptor and a heat-shock protein (both M_r 108,000) from oviducts (N. Weigel, Baylor College of Medicine, personal communication) are phosphorylated by purified PK-P.

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