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Regulation of Protein Synthesis in Plant Embryo by Protein Phosphorylation[1]

I. PURIFICATION AND CHARACTERIZATION OF A cAMP-INDEPENDENT PROTEIN KINASE AND ITS ENDOGENOUS SUBSTRATE

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ABSTRACT

A cyclic AMP-independent protein kinase, which strongly inhibits *in vitro* protein synthesis, was purified to homogeneity from barley embryo by affinity and ion exchange chromatography. The M_r of the purified enzyme is 95,000 with two nonidentical subunits of M_r 58,000 and 39,000. The enzyme activity is not stimulated by cAMP, cGMP, or calmodulin. The endogenous phosphate acceptor of this kinase is a protein of M_r 52,000, was isolated by purified protein kinase immobilized Sepharose column. Using antibodies raised against this protein kinase, the levels of the enzyme during embryogenesis and germination are determined. An inverse relationship has been observed between protein kinase level and rate of protein synthesis.

Barley embryos, like other plant embryos, contain stored mRNAs which are transcribed during embryogenesis to be utilized during germination. The translation of these mRNAs is crucial during the initial stages of germination (5, 16, 22). However, the mechanism(s) by which translation of stored mRNAs is prevented until germination is not known. In animal systems (3, 15, 17) cAMP-independent protein kinases which phosphorylate the smallest subunit (38,000 D) of eIF-2, a rate limiting initiation factor of protein synthesis, are involved in the regulation of translation. However, very little information is available about plant protein kinases and their role in protein synthesis. Ranu (19, 20) reported the partial purification of a cAMP-independent protein kinase from wheat germ which, like reticulocyte kinase, phosphorylates the 38 kD subunit of eIF-2. But it is not known whether this phosphorylation affects protein synthesis. Furthermore Rychlik *et al.* (21) reported another cAMP-independent kinase from wheat germ which selectively inhibits the translation of some species of Brome mosaic virus RNA by phosphorylating the ribosomal proteins. In this paper, we describe the purification and characterization of a novel cAMP-independent protein kinase which is a potent inhibitor of translation *in vitro*. Its endogenous substrate has been purified using a protein kinase bound Sepharose column. Using antibodies raised against the protein kinase, the level of the enzyme during different stages of development of the embryos has been correlated with the rate of protein synthesis.

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MATERIALS AND METHODS

Materials. Barley (*Hordeum vulgare*, pure line, hull-less variety IB65) was obtained from Indian Agricultural Research Institute, New Delhi. Embryos were isolated as described by Marcus *et al.* (12) using a Waring Blendor. Materials were purchased from the following sources: casein, phosvitin, histone type IIAS, protamine, BSA, heparin, cAMP, cGMP, ATP, CTP, heparin, spermine, spermidine, *p*-chloromercuribenzene sulfonic acid and Tris (Sigma); Sephadex G-100 (fine), Sepharose 6B and mol wt markers (Pharmacia, Uppsala, Sweden); UTP (Calbiochem); GTP (P-L Biochemicals, Milwaukee, WI); cyanogen bromide (Fluka A.G. Switzerland). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (7) with slight modification. Sepharose 6B was activated with cyanogen bromide (18) and then casein-Sepharose and protein kinase-Sepharose columns were constructed by the method of Thornberg and Lindell (23). Purified calmodulin from oats was kindly provided by Dr. S. K. Sopory who received it from Dr. Stanley Roux, Department of Botany, University of Texas, Austin.

Purification of Protein Kinase and Its Endogenous Substrate by Affinity Chromatography. Barley embryos (15 g) were ground in mortar and pestle with buffer A (20 mM Tris-Cl [pH 7.6], 3 mM Mg-acetate, and 1 mM DTT) and the homogenate was centrifuged at 35,000*g* for 30 min. The supernatant (1580 mg protein) was filtered through muslin cloth and applied onto a casein-Sepharose column (1.5 × 10 cm) which was previously equilibrated with buffer A. The column was extensively washed with the above buffer and bound material was eluted with 0.5 M KCl in buffer A. Peak fractions of activity (14 ml) were pooled and dialyzed. The dialyzed preparation (8.3 mg protein) was then applied onto DE52 (2.5 × 8 cm) column equilibrated with the buffer A. The column was washed with the same buffer and eluted with 100 ml 0 to 500 mM linear KCl gradient in buffer A. A minor protein peak with no enzyme activity was observed in the wash and the bound fraction eluted as single peak with enzyme activity. The specific activity of the enzyme was about 22,000 pmol ^{32}P incorporated/min·mg^{-1} protein.

The endogenous phosphate acceptor for barley protein kinase was isolated by using purified protein kinase bound Sepharose affinity chromatography. In this method, purified protein kinase was extensively dialyzed against sodium carbonate buffer, pH 9.0. The activated Sepharose 6B gel (5 ml) was suspended in 12 ml of protein kinase (4 mg protein) and stirred gently for 16 h at 0 to 4°C. Then the gel was washed with buffer A containing 0.5 M KCl to remove unbound enzyme and kept in 20 mM Tris-HCl (pH 7.6) for overnight.

The homogenate (10 ml) in enzyme buffer A of 2 g of embryos was centrifuged at 35,000*g* for 30 min. The supernatant was

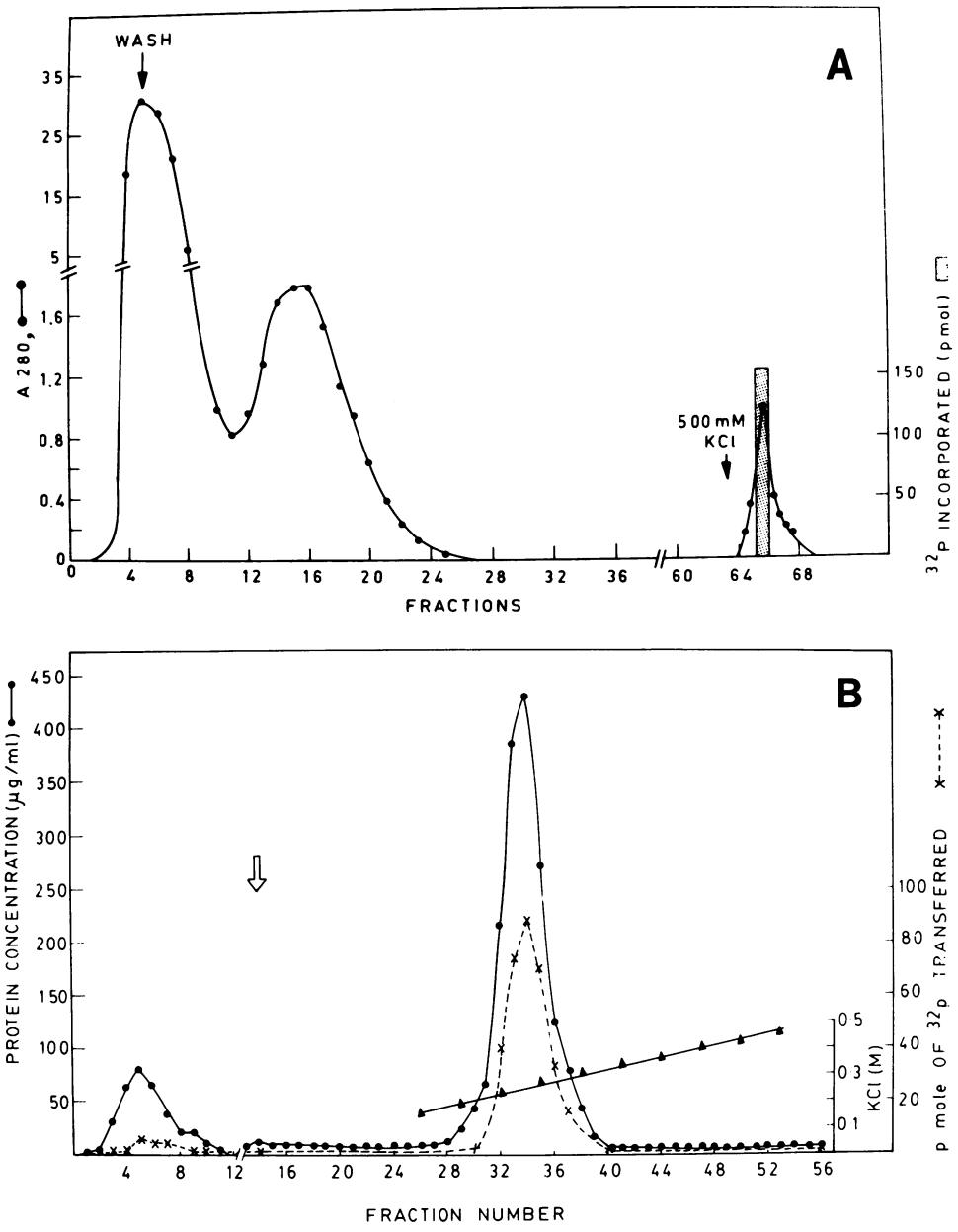

FIG. 1. Purification of protein kinase from barley embryos. A, Casein-Sepharose affinity chromatography of protein kinase. Crude extract of barley embryo (1580 mg protein) was loaded onto a casein-Sepharose column (1.5 × 10 cm). The column was then washed and eluted as described in "Materials and Methods." B, Fractions with protein kinase activity from casein-Sepharose column were pooled, dialyzed and loaded onto a DEAE-cellulose column as described in "Materials and Methods." The active enzyme was eluted with 0 to 0.5 M KCl gradient. The volume of each fraction was 2.5 ml. The arrow indicates the start of the gradient.

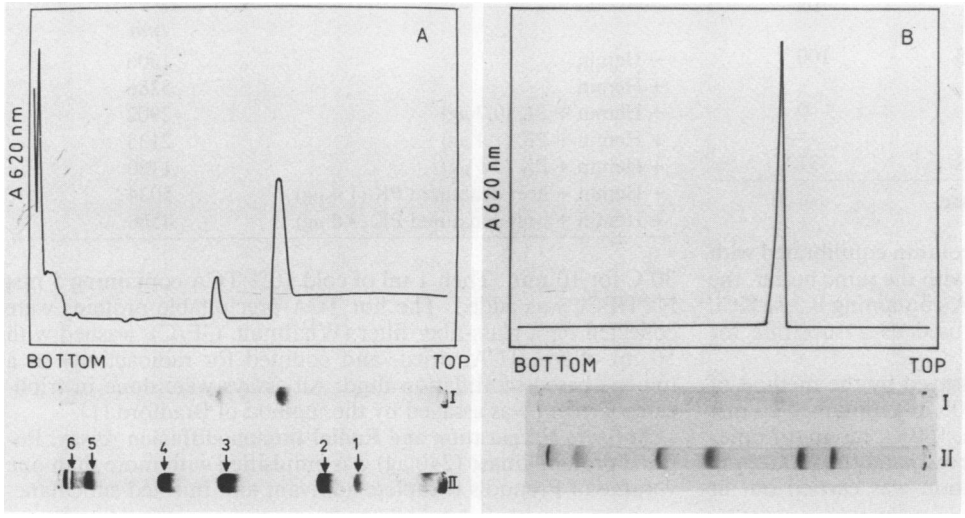

FIG. 2. Densitometric scan of SDS-PAGE of protein kinase (A) and endogenous substrate (B). Ten cm long 10% polyacrylamide gels containing 0.1% SDS with 1 cm 3% stacking gels were run at 3 mamp per tube by the method of Laemmli *et al.* (11). Lane I: Protein kinase (A) or substrate (B). Lane II: Mol wt markers 1, phosphorylase (94,000); 2, BSA (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, soybean trypsin inhibitor (20,100); 6, α-lactalbumin (14,000).

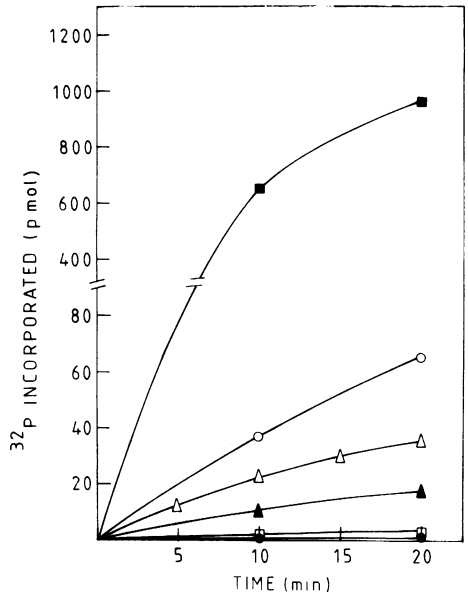

FIG. 3. Substrate specificity of protein kinase. Protein kinase was used for the assay as described in "Materials and Methods." One hundred μg of endogenous substrate (■), casein (△), histone (▲), phosvitin (○), protamine (□), BSA (●) were used per assay. Assay was done according to the procedure described in "Materials and Methods."

Table I. *Properties of Protein Kinase*

Component in Assay	Enzyme Activity	Percent Control
	pmol ^{32}p transferred/min	
Complete system	19.08	100
−Protein kinase	0	0
−Substrate	0	0
Additions		
Cyclic AMP		
5.10^{-6} M	18.2	95.4
1.10^{-5} M	16.08	84.3
Cyclic GMP		
5.10^{-6} M	17.36	91.0
1.10^{-5} M	16.44	86.1
Ca^{+2}		
5.10^{-5} M	19.0	100
5.10^{-4} M	18.85	99
CAM[a] (1 μg)	19.5	103
Ca^{+2} (5.10^{-5} M) + CAM (1 μg)	19.4	102
Ca^{+2} (5.10^{-4} M) + CAM (1 μg)	19.05	100
Heat-treated enzyme (70°C, 10 min)[b]	0	0
p-Chloromercuric-benzene, sulfonic acid (1 mM)	6.08	31.8

[a] Calmodulin. [b] Instead of protein kinase.

applied onto enzyme bound Sepharose column equilibrated with the buffer A. After washing the column with the same buffer, the bound material was eluted with buffer A containing 0.5 M KCl. The eluted protein was dialyzed and used as a substrate for protein kinase.

Enzyme Assay. Protein kinase was assayed by the method of Datta *et al.* (4). The reaction mixture (0.05 ml) contained 20 mM Hepes-KOH (pH 7.6), 5 mM Mg-acetate, 100 μg casein (or other substrates), 35 to 60 μM [γ-^{32}P]ATP (approximately 60–100 cpm/pmol) and protein kinase. The incubation was carried out at

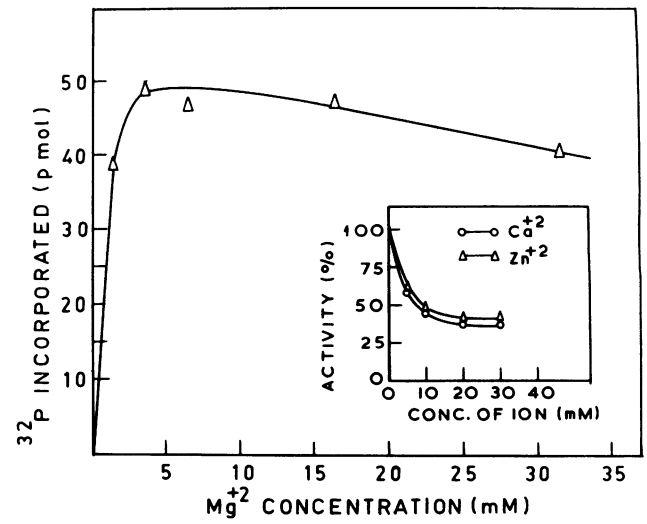

FIG. 4. Effect of varying concentrations (0–30 mM) of Mg^{2+} on the activity of protein kinase. The inset shows the effect of Ca^{2+} and Zn^{2+} on protein kinase activity in presence of 1.5 mM Mg^{2+}.

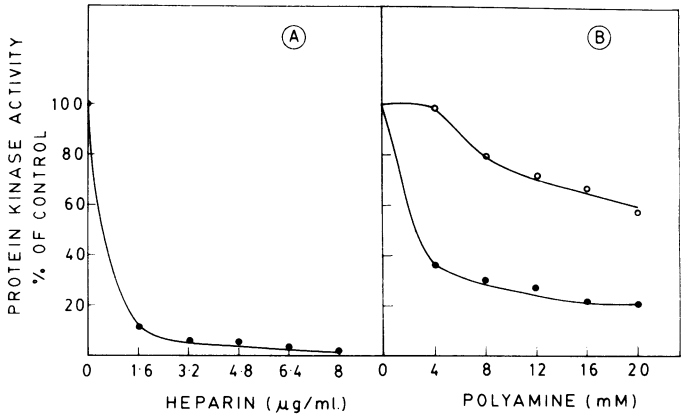

FIG. 5. Effect of (A) heparin and (B) polyamines: (●) spermine, and (○) spermidine on activity.

Table II. *Effect of Protein Kinase (PK) on in Vitro Translation System*

Translation assays were done in a final volume of 100 μl in presence or absence of 30 μM hemin. After 20 min, incubation at 30°C, 25 μl aliquots were removed, precipitated, and incorporation was determined. Enzyme was denatured by heating at 90°C for 5 min.

System	Incorporation of [^{14}C]Lysine
	cpm
− Hemin	1306
+ Hemin	5386
+ Hemin + PK (0.7 μg)	2902
+ Hemin + PK (1.4 μg)	2133
+ Hemin + PK (3.6 μg)	1120
+ Hemin + heat denatured PK (1.4 μg)	5024
+ Hemin + heat denatured PK (3.6 μg)	4566

30°C for 10 min. Then 1 ml of cold 10% TCA containing 5 mM Na_2HPO_4 was added. The hot TCA precipitable proteins were collected on a glass-fiber filter (Whatman, GF/C), washed with 50 ml of 5% TCA, dried, and counted for radioactivity in a toluene-based scintillation fluid. All assays were done in triplicates. Protein was assayed by the method of Bradford (1).

Antisera Preparation and Radial Immunodiffusion Assay. Purified protein kinase (240 μg) was emulsified with more than one volume of Freund's complete adjuvant and injected subcutane-

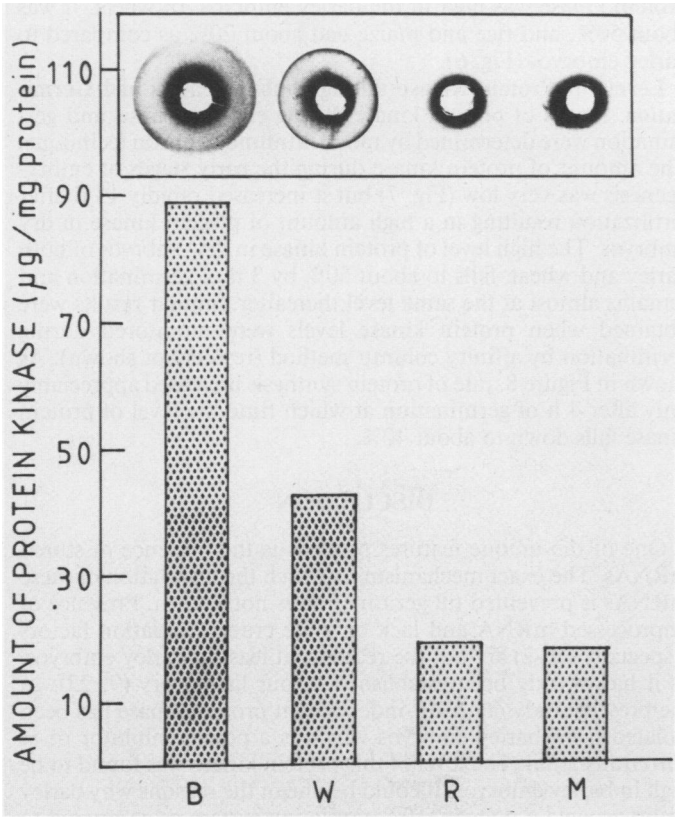

FIG. 6. Radial immunodiffusion assay of protein kinase using 75 µg protein from mature embryos of barley (B), wheat (W), rice (R), and maize (M).

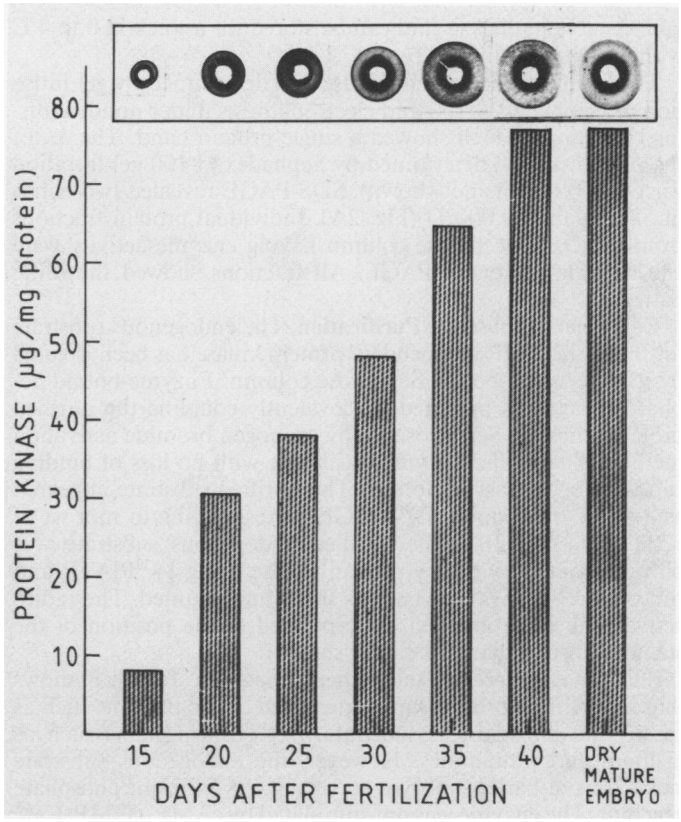

FIG. 7. Levels of protein kinase during embryogenesis of barley embryos. One hundred mg embryos, from seeds collected on different days after fertilization were homogenized and the extract (75 µg protein) was used to determine the level of the protein kinase by radial immunodiffusion assay. Inset: radial immunodiffusion patterns of protein kinase of different embryos.

ously at multiple sites on the back of a New Zealand White rabbit. A booster injection of 160 µg was given after 1 month. Rabbit was bled 15 d later and serum was separated. The presence of antibodies was checked by Ouchterlony immunodiffusion (6).

Radial immunodiffusion was carried out on glass slides (75 × 25 cm) coated with 1% agarose in borate buffer (6.18 g boric acid, 9.53 g Borax, 4.38 g NaCl, and distilled H_2O to 1 L, pH 8.4–8.5) with antisera mixed in agarose in proportion of 1:40 (v/v) (6). Wells were made on these agarose plates and 15 µl of the extract (75 µg) were loaded in these wells. Simultaneously, a standard was also run with known amounts of pure protein kinase. The slides were incubated in a moist chamber at 37°C for 48 h, washed in 1% saline, dried, stained with 0.1% Coomassie brilliant blue R-250 in acetic acid: ethanol:water (10:45:45), then destained with acetic acid:ethanol:water (10:25:60). A calibration curve was prepared with the known values of protein kinase and used to measure the actual antigen concentration of different embryo extracts.

RESULTS

Protein Kinase Purification. A cyclic AMP-independent protein kinase has been purified from barley embryos by column chromotography on casein-Sepharose and DEAE cellulose (Whatman DE52). The cytosolic crude extract was applied onto a casein-Sepharose column, and bound proteins were eluted with 0.5 M KCl. As shown in Figure 1A, a single protein peak having enzyme activity was eluted. The active fractions were pooled and loaded onto a DEAE-cellulose column. In the wash a minor protein peak without protein kinase activity appeared. As shown in Figure 1B, the bound proteins were eluted as one major peak (0.2–0.3 M KCl). The specific activity of this enzyme was 21,900 pmol of ^{32}P transferred/min·mg^{-1} protein. The enzyme is heat

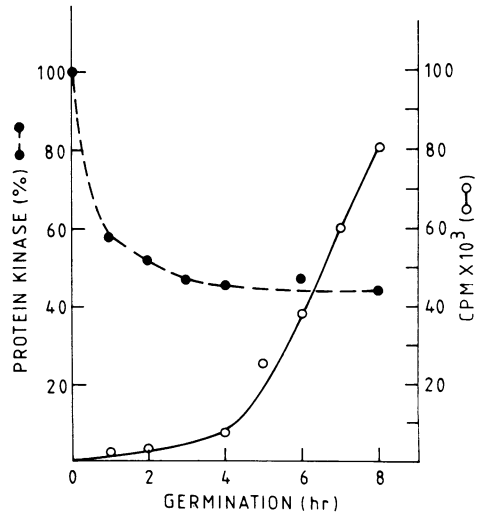

FIG. 8. Rate of protein synthesis and level of protein kinase during germination. At the indicated times, embryos (15 mg) were labeled with [^{3}H]leucine (40 µCi/ml) for 1 h. They were washed, homogenized in buffer, A, precipitated with TCA, filtered and the radioactivity was determined. (○) rate of protein synthesis, (●) protein kinase levels as determined by radial immunodiffusion method.

labile, stable to dialysis and can be stored for a week at 0 to 4°C without loss of activity.

The purity of the protein kinase was determined by gel filtration on Sephadex G-100 and electrophoresis under nondenaturing conditions, which showed a single protein band. The M_r of the protein kinase determined by Sephadex G-100 gel filtration was 95,000 (results not shown). SDS-PAGE revealed two bands of 58,000 and 39,000 D (Fig. 2A). Individual protein fractions from the DEAE cellulose column having enzyme activity were checked for purity on PAGE. All fractions showed the same pattern.

Endogenous Substrate Purification. The endogenous substrate for the cyclic AMP-independent protein kinase has been isolated by using enzyme-bound Sepharose column. Enzyme-bound Sepharose has been prepared by covalently coupling the purified protein kinase to Sepharose 6B by cyanogen bromide activation method. The bound enzyme was stable with no loss of binding capacity for at least 3 months. The purified substrate appeared as a single band upon SDS-PAGE corresponding to mol wt of 52,000 D (Fig. 2B). The purified endogenous substrate was phosphorylated by barley protein kinase using [γ^{32}P]ATP and run on SDS-PAGE. The gel was sliced and counted. The radioactive peak thus obtained corresponded to the position of the stained substrate band (data not shown).

Substrate Preference and Other Properties. Figure 3 shows that the purified protein kinase preferred acidic proteins such as casein and phosvitin as substrate over basic proteins such as histones and protamines. However, the endogenous substrate isolated from barley embryos served as very efficient phosphate acceptor. The enzyme was not stimulated by cAMP, cGMP, Ca^{2+} or calmodulin (Table I). It showed no autophosphorylation in the absence of added substrate. The K_m value for ATP was 12.5 μM with a fixed concentration of casein. As shown in Figure 4, the optimum Mg^{2+} concentration was 4 mM. Increasing concentrations of Mg^{2+} up to 30 mM did not have much effect on the activity. However, the activity of the enzyme was markedly inhibited when assayed in presence of Ca^{2+} or Zn^{2+} (Fig. 4). No aggregation was observed with Ca^{2+} or Zn^{2+} during incubation.

Since the activity of casein phosphorylating protein kinase is modulated by heparin and polyamines, the effect of these on the enzyme activity was determined. As shown in Figure 5, about 90% inhibition of enzyme activity was observed at a low heparin concentration of 1.6 μg/ml. Polyamines, *i.e.* spermine and spermidine, are known to stimulate casein kinase II (10, 13, 14). But, as shown in Figure 5, the barley protein kinase was markedly inhibited by polyamines. These results suggest that the barley protein kinase is different from other reported kinases.

Effect of Protein Kinase on Protein Synthesis. As shown in Table II, this protein kinase strongly inhibited translation in hemin-containing reticulocyte lysates. The degree of inhibition increases with increasing concentrations of protein kinase. However, the translation inhibition was abolished when the enzyme was heated for 5 min at 90°C which also destroys the protein kinase activity.

Cross Reactivity of Extracts with Antiserum. Antibodies against protein kinase were used to study the presence of the enzyme in different plants and tissues and to determine its level in barley embryos during embryogenesis and germination. We have observed that embryo extracts of monocotyledonous seeds (barley, wheat, maize, and rice) cross-reacted with antiserum raised against barley protein kinase, whereas embryo extracts of dicotyledonous seeds (pea and *Cajanus* embryos) did not cross react with the antiserum. Extracts of endosperm, leaves, reticulocyte lysate, and yeast also did not give a positive response (results not shown). These results suggest that this protein kinase is localized in the embryos of monocotyledonous seeds. Moreover, radial immunodiffusion studies showed that the level of protein kinase was high in the barley embryos. In wheat, it was about 50%, and rice and maize had about 20% as compared to barley embryos (Fig. 6).

Levels of Protein Kinase during Embryogenesis and Germination. Levels of protein kinase during embryogenesis and germination were determined by radial immunodiffusion technique. The amount of protein kinase during the early stages of embryogenesis was very low (Fig. 7) but it increased rapidly 15 d after fertilization resulting in a high amount of protein kinase in dry embryos. The high level of protein kinase in dry embryos of both barley and wheat, falls to about 50% by 3 h of germination and remains almost at the same level thereafter. Similar results were obtained when protein kinase levels were monitored during germination by affinity column method (results not shown). As shown in Figure 8, rate of protein synthesis increased appreciably only after 4 h of germination at which time the level of protein kinase falls down to about 40%.

DISCUSSION

One of the unique features of seeds is the presence of stored mRNAs. The exact mechanism by which the translation of these mRNAs is prevented till germination is not known. Presence of unprocessed mRNA and lack of some crucial initiation factors (especially eIF-2) are not the reasons, at least in barley embryos, as it has already been established in our laboratory (9, 22). In the present study, a cAMP-independent protein kinase has been isolated from barley embryos which is a potent inhibitor of *in vitro* translation. The level of this protein kinase was found to be high in barley embryos. It could be one of the reasons why barley embryos yield a poor *in vitro* translation system as compared to wheat or rice (2) where the level of this enzyme is very low (Fig. 6). In addition it was found to be localized only in the embryo, which strongly suggests that it plays a role in the development of the plant.

It is known that after fertilization, the synthesis of RNA and protein is quite high during the first 15 d and slows down thereafter (8). Seeds reach a maximum weight by the 30th d after which they dehydrate. The dehydration phase is completed by the 40th d (9). Most synthetic processes, including protein synthesis, would probably be completed by the 30th d. The suppression of translation due to the presence of high level of protein kinase, synthesized during late stages of embryogenesis (20–35 d), could be the mechanism by which some mRNAs are conserved for use during germination. On germination, the level of protein kinase falls to about 40% by 4 h. As shown in Figure 8, the rate of protein synthesis increased appreciably 4 h after germination; at this time the level of protein kinase is low. Hence, there is an inverse relationship between protein kinase level and protein synthesis, indicating this enzyme might have a role in preventing translation of stored messages.

Endogenous substrate of this protein kinase has also been purified to homogeneity by a novel method, *i.e.* protein kinase bound Sepharose column (Fig. 2B). The possibility of its involvement in the initiation step of translation cannot be ruled out since protein kinase inhibits initiation of protein synthesis. Further work is in progress to understand the molecular mechanism of inhibition of protein synthesis and the role of endogenous substrate in translation.

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