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A HEAT-STABLE Mg-INDEPENDENT PROTEIN KINASE OF GERMINATED BARLEY SEEDS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; protein kinase; autophosphorylation; protein phosphorylation.

Abstract—A protein kinase that is fully active in the presence of EDTA and resists temperatures up to 70° has been detected and partially purified from the 105 000 *g* supernate of barley seeds germinated in the absence of light for 84 hr. The enzyme is excluded from a Sephadex G-75 column and is autophosphorylated on a M_r 11 500 subunit. Enzyme activity shows two optima: one at pH 5.5–6.0 and another at pH 7.5.

INTRODUCTION

Since the realization over the past six or seven years that protein kinases play a pivotal role in the control of cellular metabolism, an ever-increasing number of workers has been drawn into this field. In fact Hunter, who called these enzymes the 'transistors of the cell' pointed out that over the first 20 years since the discovery of the first protein kinase in 1959 there were known only about 10 mammalian enzymes while over the past seven to eight years the number has increased to over 70 [1]. In contrast, as far as we were able to find out, less than a dozen plant protein kinases have been purified either partially or to homogeneity (e.g. [2–5]. Even so, there have been reports for protein phosphorylation patterns in a number of plant tissues (e.g. [6–9]). An even more obscure topic in plant biochemistry is the regulation of the ATP: protein phosphotransferase reaction. There are few reports [10–12] of the functioning of cyclic AMP-dependent protein kinases in plants. Ca^{2+}-Dependent protein phosphorylation has been reported in pea shoot membranes [13] wheat germ [14] and amyloplasts of cultured cells of sycamore [9].

In the present work we studied the protein phosphorylating potential of dormant as well as germinated barley seeds and detected in the latter an interesting protein kinase that is relatively heat stable and operates in the absence of free metal ions.

RESULTS

The kinetics of incorporation of phosphate from the γ position of ATP into trichloracetic acid insoluble products of S_{105} are linear over a period of 4–5 min when the assay mixture contains *ca* 50 μg protein. As the reaction progresses, incorporation starts decreasing, apparently due to the presence of residual activity from the large number of acid phosphatases present in germinated barley [15]. Cyclic-AMP does not significantly affect the rate of phosphorylation. As shown in Fig. 1 two groups of proteins are phosphorylated in the soluble fraction of germinated barley: a high M_r group (M_r *ca* 60–70 000) and a low M_r group (M_r *ca* 11–14 000). The high M_r group contains at least two-polypeptides with a M_r of 64 and 70 000 respectively, while the low M_r group also contains at least two polypeptides of 14 and 11 500 respectively. Ungerminated seeds lack the low M_r polypeptides but are particularly rich in the high M_r group. Germination in the presence of light does not seem to affect the overall phosphorylation pattern. Neither is the pattern changed when incubations with ATP are performed in the presence of 0.3 mM phenyl methane sulphonyl fluoride, a potent inhibitor of serine proteinases. Roots lack the high M_r group of phosphorylated proteins but are particularly rich in the low M_r group and especially the 14 000 polypeptide. The latter is the only phosphorylated peptide produced upon incubating isolated chromatin with ATP (result not shown). In addition repeated freeze–thaw cycles destroy or denature both the high and the low M_r polypeptides, and finally the phosphorylated products of both high and low M_r are sensitive to proteinase K (Fig. 1). Treatment of gels with alkali leads to the disappearance of all radioactive bands (results not shown). Cooper *et al.*, who proposed the treatment, have shown that the monoesterified phosphate of phosphotyrosine-containing peptides is more alkali stable than the respective phosphates of phosphoserine and phosphothreonine-containing peptides [16]. The result is an indication that the protein kinases responsible are not of the tyrosine type but rather of the serine/threonine type. The curve of the heat sensitivity of protein kinase activity in the S_{105} fraction of germinated barley seeds shows a shoulder at *ca* 70° (Fig. 2) suggesting the probable presence of a heat-resistant kinase. This is indeed the case, S_{105} heated to 70° phosphorylates the M_r 11 500 polypeptide (Fig. 3).

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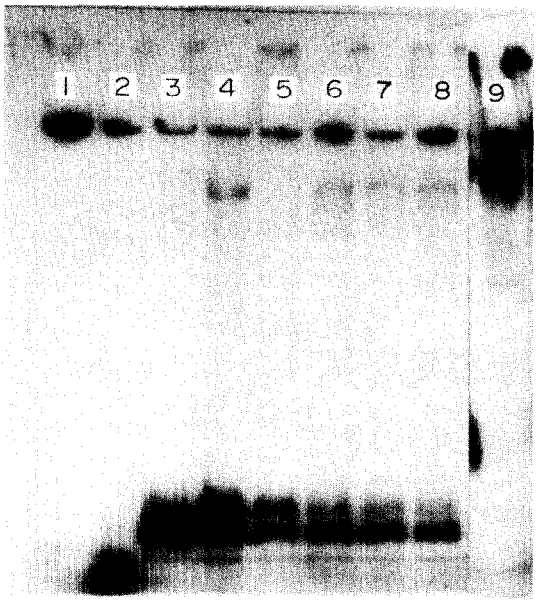

Fig. 1. Autoradiograms of polyacrylamide slab gels after SDS-electrophoresis of the following mixtures 1. M_r markers plus {γ-^{32}P}ATP. 2. S_{105} of total seeds followed incubation with 16 μg/ml proteinase K. 3. S_{105} of total seeds subjected to three freeze-thaw cycles. 4. S_{105} of first leaves. 5. S_{105} of roots. 6. S_{105} of total seeds germinated in the dark. 7. S_{105} of total seeds germinated under artificial light. 8. S_{105} of total seeds germinated in light–dark cycles. 9. S_{105} of ungerminated seeds. All mixtures were incubated for 2 min. Details for incubation conditions and SDS-polyacrylamide gel electrophoresis are given in the Experimental section. Column 9 is from a different autoradiogram than the rest of the columns.

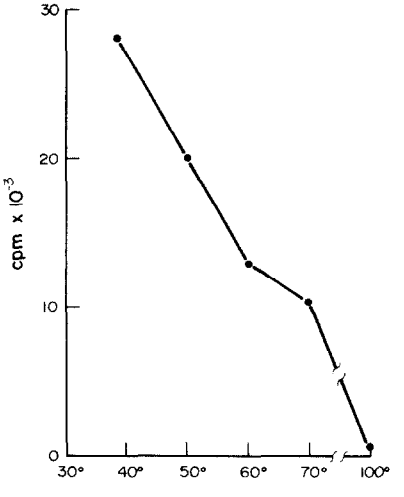

Fig. 2. Effect of heating the S_{105} fraction at the indicated temperatures for 10 min. Each heated preparation was assayed for protein kinase activity as described in the Experimental section.

The same protein is phosphorylated when EDTA is included in the reaction mixture. Adding various amounts of Mg^{2+} to enzyme preparations dialysed against 10^{-4} M EDTA led to no more than 20% increase in activity with 10^{-3} M Mg^{2+}, while higher concentrations led to inhibition. Ca^{2+}, Mn^{2+} and Cu^{2+} at concentrations of 10^{-3} and 10^{-2} M were also ineffective. Consequently there is a protein kinase resistant to heating to 70° that does not require exogenous bivalent metal. In order to establish whether the metal independent kinase is a self-phosphorylating enzyme, we proceeded as follows: the S_{105} fraction was heated to 70° and was subjected to polyacrylamide gel electrophoresis under non-denaturing conditions both following incubation with (γ-^{32}P) ATP in the presence of EDTA as well as without incubation. In the first case the gel was sliced into 3 mm portions and their radioactivity was counted. In the second case the gel was sliced into 3 mm portions and each portion was assayed for protein kinase activity in the presence of EDTA. In each gel one peak of radioactivity appeared. Both peaks migrated exactly the same distance. Since the substrate has exactly the same mobility as the enzyme, there is a strong probability that we are dealing with an autophosphorylating protein kinase. Gel permeation chromatography of S_{105} heated to 70° through a Sephadex G-75 column reveals the presence of one autophosphorylating activity that appears in the void volume. Heat treatment and gel permeation chromatography in-

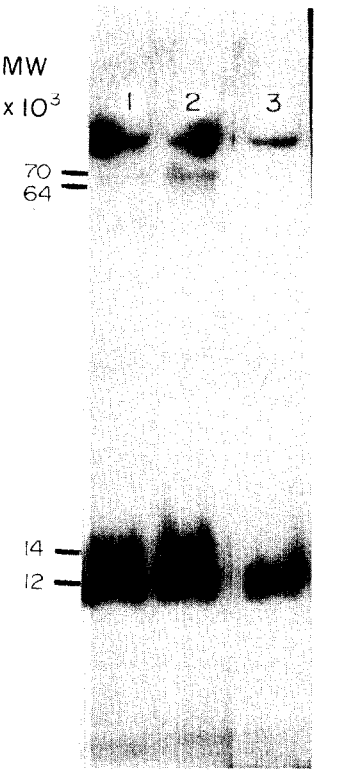

Fig. 3. Autoradiograms of polyacrylamide slab gels after SDS-electrophoresis of the following mixtures: 1. S_{105} previously dialysed against 50 volumes 75 mM Tris–acetate pH 7.5 and resupplied with 1 mM $MgCl_2$. 2. S_{105} 3. S_{105} heated to 70° for 10 min or dialysed prior to incubation and not resupplied with Mg^{2+} or incubated in the presence of EDTA. All mixtures were incubated for 2 min. Details for incubation conditions and SDS-polyacrylamide gel electrophoresis are given in the Experimental section. Column 3 is from a different autoradiogram than columns 1 and 2.

crease enzyme activity by at least one order of magnitude (Table 1) The metal-independent kinase can be further purified by adsorption onto a DEAE-cellulose column at pH 8.5 in the presence of 20 mM Tris–acetate and elution by means of a 0–400 mM NaCl gradient in the same buffer (250 ml for a 22 × 1 cm column). The pH-activity curve of the purified preparation is shown in Fig. 4. The enzyme shows two optima: one at pH 5.5–6.0 and another at pH 7.5. The enzyme cannot phosphorylate histone, protamine, bovine serum albumin or dephosphorylated casein when 100 μg of any of these proteins are included in the assay mixtures. Finally the enzyme is not activated by cyclic AMP when the latter is added to the assay mixture up to a concentration of 10^{-5} M.

DISCUSSION

The protein kinase studied in the present work has a number of properties not ordinarily found in the respective mammalian enzymes. Firstly, it is fully active in the presence of EDTA. As far as we know this is the only kinase reported that does not require Mg^{2+}. It may be that the metal is bound very tighly to the enzyme and cannot be chelated by EDTA. Careful analysis for the presence of protein-bound metal ions will have to be performed when the enzyme is obtained in pure form. The second unusual property is its pH-activity curve with 2 optima. Lowest activity is evident at the pK value of the secondary phosphate. As far as we know, the bovine rhodopsin kinase is the only other protein kinase that shows two pH optima [17]. The relative heat stability of the enzyme is a third unusual feature. The Mg^{2+}-dependent kinase(s) of barley lose all activity when heated to 70°. The resistance of the metal-independent kinase to temperatures up to 70° also helps in its purification since a large number of proteins are denatured at this temperature. In addition one or more inhibitors are removed by the heat and the gel filtration treatment of the preparation. The inhibitory substance(s) apparently suppress enzyme activity by more then 90%. Finally the enzyme is autophosphorylated in a small subunit of M_r 11 500. At this stage of the investigation we do not know whether autophosphorylation affects enzyme activity.

Table 1. Increase in total and specific activity of the metal-independent kinase following heat treatment and gel permeation chromatography of the S_{105} fraction

Treatment of S_{105}	Volume (ml)	Total activity (cpm)	Total protein (μg)	Specific activity (cpm/μg protein)
None	2.5	319000	4500	70
Heated to 70°	2.5	1326000	2250	590
Heated to 70° and filtered through Sephadex G-75	3.5	4437000	175	25400

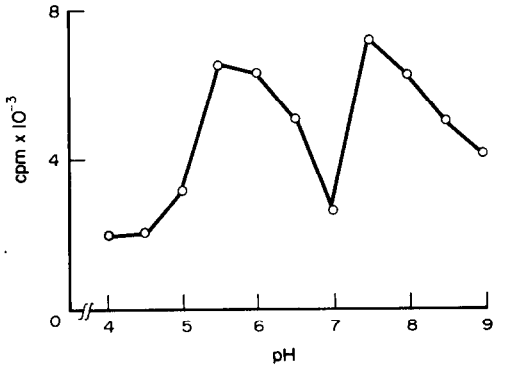

Fig. 4. Effect of pH on autophosphorylation of S_{105} filtered through a column of Sephadex G-75. 2.5 μg of enzyme preparation were incubated for 2 min at 37° in a total volume of 70 μl with 0.125 pmol {γ-^{32}P}ATP (6000 Ci/mmol) and 93 mM ammonium acetate (pH 4–6.5) or Tris–acetate (pH 7–9). The same form of curve is obtained in the pH region 6–8 when 93 mM K-Pi buffer is used.

EXPERIMENTAL

All biochemical reagents were purchased from Sigma, (γ^{32}-P) ATP (6000 Ci/mmol) was a product of New England Nuclear.

Seeds of *Hordeum vulgare*, var. Georgia were used throughout this work. They were generously provided by the Cereal Institute of Thessaloniki. Prior to germination the seeds were treated as described in [15]. Germination took place at 21.5 ± 1° in the absence of light for 84 hr over wet cotton pads and the germinated seeds were placed at −30° for at least 84 hr. Henceforth all operations took place at 0–4°. For the preparation of the crude extract the frozen material was homogenized in 3 vol 0.1 M Tris–acetate buffer pH 7.0 for 3 min at 10000 rpm in an Omni-Mixer. The homogenate was filtered through 4 layers of surgical gauze and the filtrate was centrifuged at 13 500 g for 15 min. The supernate was centrifuged at 105000 g for 1 hr and the new supernate (S_{105}) was used as a source of enzyme activity. Whenever enzyme activity was tested separately in roots and leaves, prior to homogenization, the respective parts of the plant were triturated with sand in a pre-cooled mortar and pestle. Chromatin was prepared by the method of ref. [18]. Disc electrophoresis under nondenaturing conditions was performed in 80 × 5 mm cylinders of 7.5% polyacrylamide gels at pH 8.5 as described in [19].

Electrophoresis in 12 × 16 × 0.15 cm polyacrylamide slab gels in the presence of SDS and autoradiography of the electrophorograms were performed as described in [20]. Protein kinase activity was assayed by incubating 25 μl of the enzyme preparation in a total vol. of 50 μl at 37° for 2 min in the presence of 0.1 pmol (γ-^{32}P) ATP, 33 mM Tris–HCl pH 7.6, 1 mM Mg^{2+} or 1 mM EDTA and 10 mM K-Pi pH 7.5. At the end of the incubation period 40 μl aliquots were placed on Whatman 3 MM filter papers of 3.8 cm diameter which were successively dipped in the following solns for the respective time periods: (a) 10% trichloroacetic acid in 10 mM pyrophosphate for 20 min, (b) 5% trichloroacetic acid in 10 mM pyrophosphate for 20 min, (c) same soln as in (b) but for 10 min. The filter papers were finally washed in EtOH and Et_2O and their radioactivity counted in a liquid scintillation counter, dipped in a toluene-based scintillation cocktail.

In this paper protein kinase activity is expressed in terms of radioactivity (cpm) incorporated into TCA insoluble products over a period of 1 min.

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