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SOME PROPERTIES OF STARCH PHOSPHORYLASE FROM COTYLEDONS OF GERMINATING SEEDS OF *VOANDZEIA SUBTERRANEA*

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Summary

Two isoenzymes (Forms I and II) of starch phosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1) were found in cotyledons of germinating seeds of *Voandzeia subterranea* L. Thouars. Phosphorylase I, which was the major component, had a pH optimum of 5.5—5.6, whereas phosphorylase II had a pH optimum of 6.1—6.3. Phosphorylase I had a molecular weight of 204 000 ± 4000 and a subunit molecular weight of about 95 000. Phosphorylase I was stimulated by Mg^{2+}, Mn^{2+}, AMP, cyclic AMP, pyruvate and EDTA, but inhibited by Fe^{2+}, Cu^{2+}, Zn^{2+} and ATP. Stimulation of phosphorulase I by AMP was accompanied by changes in the affinity of the enzyme for glucose-1-phosphate in the presence of increasing AMP concentrations, and of AMP in the presence of increasing glucose-1-phosphate concentrations.

Double-reciprocal plots of initial velocity data were non-linear (convex up) at low glucose-1-phosphate concentrations but became linear in the presence of AMP or ATP. Double-reciprocal plots were linear at high glucose-1-phosphate concentrations in the absence or presence of modifiers.

Introduction

Starch phosphorylase is classified with glycogen phosphorylase as 1,4-α-D-glucan:orthophosphate α-glucosyltransferase (EC 2.4.1.1.). Detailed studies on starch phosphorylase have been restricted to potato phosphorylase. Although the potato enzyme is similar to glycogen phosphorylase in many respects, it differs from glycogen phosphorylase in that it is not stimulated by AMP, lacks any homotropic cooperativity and cannot be converted into a tetramer by

enzyme-catalysed phosphorylation [1—6]. Multiple forms of starch phosphorylase have been isolated from maize endosperm, and these are also not stimulated by AMP but are inhibited by ATP [7,8]. Two forms of the enzyme have been found in *Oscillatoria princeps*, and only one of these was found to require AMP for activity [9]. Multiple forms of starch phosphorylase have also been found in a variety of plant tissues [10—12].

Preliminary experiments indicated that starch phosphorylase activity increases substantially in the cotyledons of *Voandzeia subterranea* (Bambarra groundnut) during germination, when the activities of amylases have declined. In view of the insensitivity of starch phosphorylase from potato and maize endosperm to AMP, some properties of the major isoenzymes found in cotyledons of *V. subterranea* during germination have been studied in order to extend available information on plant starch phosphorylase.

Materials and Methods

Reagents and chemicals. ATP, α-D-glucose-1-phosphate (glucose-1-phosphate), pyruvic acid (sodium salt), adenosine 5′-monophosphate (AMP), adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and β-galactosidase were obtained from Sigma Chemical Company. Sephadex G-200 and Blue Dextran were purchased from Pharmacia and diethylaminoethyl cellulose (DEAE-cellulose) type DE52 was a product of Whatman. Bovine serum albumin and β-lactoglobulin were obtained from Koch-Light Laboratories and catalase (beef liver) and aldolase (rabbit muscle) were products of Boehringer. All other chemicals and reagents were standard commercial products of analytical grade.

Analytical methods. Ion-exchange chromatography was performed on a column of DEAE-cellulose prepared and equilibrated with 0.01 M Tris-HCl buffer (pH 7.0), and the elution of applied protein was effected with a linear gradient of NaCl prepared from 200 ml of the plain buffer and 200 ml of the buffer containing 1 M NaCl. Molecular weights were estimated by gel-filtration on a column of Sephadex G-200 with 0.01 M Tris-HCl buffer (pH 7.0) as eluting buffer and with catalase (mol. wt. 240 000), aldolase (mol. wt. 158 000), bovine serum albumin (mol. wt. 67 000) and β-lactoglobulin (mol. wt. 35 000) as protein markers [13,14]. Subunit molecular weight was determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis with myosin (subunit mol. wt. 220 000), β-galactosidase (subunit mol. wt. 130 000), serum albumin (subunit mol. wt. 67 000) and catalase (subunit mol. wt. 60 000) as described by Weber and Osborn [15]. Polyacrylamide gel electrophoresis in the absence of SDS was carried out as described by Ornstein and Davis [16]. Protein was determined by the method of Lowry et al. [17] with bovine serum albumin as standard.

Germination of seeds and enzyme extraction. Seeds of *V. substerranea* (white seed coat variety) were planted in soil, contained in wooden boxes, away from light in a greenhouse and watered every morning. The seedlings were harvested after 2 and 8 days, and the cotyledons were removed and washed several times with distilled water. About 42 g of the blotted cotyledons were ground into a paste with 20 ml of cold 0.01 M Tris-HCl buffer (pH 7.0) in a pre-cooled mortar. The paste was then squeezed through 3 layers of cheese cloth

and the homogenate was then centrifuged at 10 000 × *g* for 40 min at 4°C. The supernatant solution (crude extract) was stored frozen until required for further studies.

Phosphorylase assay. The standard assay technique used was a modification of that of Lee [3]. The assay mixture contained, in a final volume of 1 ml, 0.05 M citric acid/sodium citrate buffer (pH 5.7), 2 mM glucose-1-phosphate, 0.1% (w/v) soluble starch and 0.1 ml of the enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 0.5 ml of 5% trichloroacetic acid. The pH was then adjusted to about 4.0 by the addition of 3 ml of 0.1 M sodium acetate solution. The amount of inorganic phosphate released was then estimated as described by Plummer [18]. Two reagent blanks were always included; one without the enzyme and the other without glucose-1-phosphate. Enzyme activity was expressed as μg P_i liberated/min. The concentration of reagents were final concentrations in the assay mixtures (1 ml).

Results and Discussion

Physical properties

$(NH_4)_2SO_4$ fractionation of the crude extract of cotyledons of *V. subterranea* that were harvested after 8 days of germination showed that the bulk of starch phosphorylase activity was precipitated at 25—50% saturated $(NH_4)_2SO_4$.

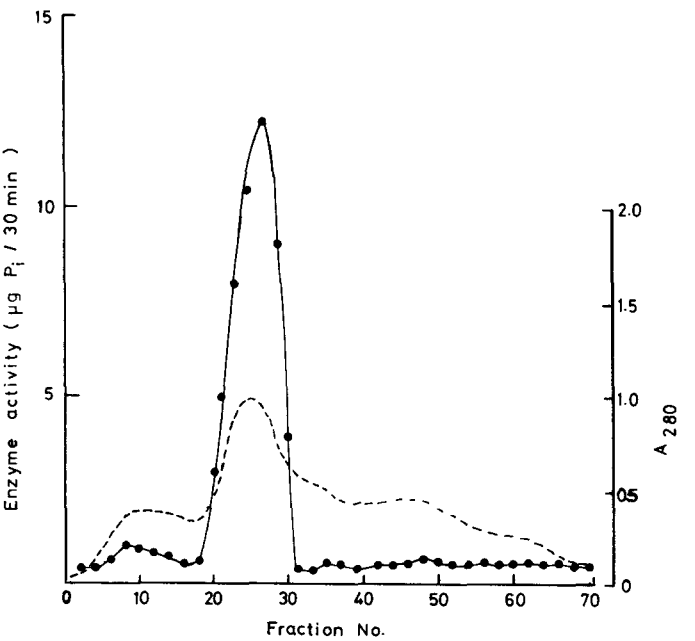

Fig. 1. Gel filtration of a solution of the 25—50% saturated $(NH_4)_2SO_4$ precipitate from the crude extract of cotyledons of *V. subterranea* obtained after 8-days germination on a column of Sephadex G-200 (2.4 × 50 cm). The eluting buffer was 0.01 M Tris-HCl buffer containing 0.1 M NaCl (pH 7.0), and each fraction was 3.0 ml. Protein distribution was determined as absorbance at 280 nm (- - - - - -) and starch phosphorylase activity (●) was determined.

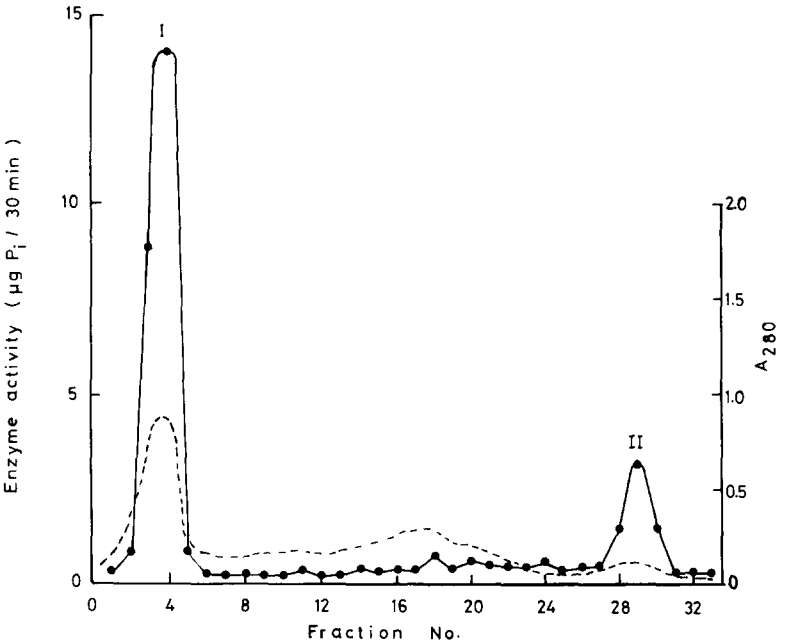

Fig. 2. Ion-exchange chromatography of pooled and concentrated fractions 20—30 in Fig. 1 on a column (1 cm × 20 cm) of DEAE-cellulose, DE-52, with a linear gradient of NaCl. The NaCl gradient was prepared from 200 ml 0.01 M Tris-HCl (pH 7.0) and 200 ml of the same buffer containing 1 M NaCl. •, Starch phosphorylase activity; and ------, protein distribution as absorbance at 280 nm. Starch phosphorylase I was eluted at about 0.05 M NaCl and starch phosphorylase II at about 0.3 M NaCl.

The results of gel-filtration on a column of Sephadex G-200 of a solution of one such protein precipitate is shown in Fig. 1. Fractions 20—30 in Fig. 1 were pooled, and protein was precipitated with 2 vols. of cold acetone. The precipitated protein was removed by centrifugation and dissolved in a minimal amount of 0.01 M Tris-HCl buffer, pH 7.0. After dialysis, a portion (4 ml) of the solution was applied to a column of DEAE-cellulose (DE-52) and elution was effected with a NaCl gradient. The results (Fig. 2) showed two enzymically active peaks. The major peak, which was eluted first, is designated phosphorylase I and the minor peak phosphorylase II. By comparing the elution profiles of extracts of cotyledons harvested after 2 and 8 days of germination, it was found that the increase in starch phosphorylase activity during germination was due to an increase in the level of phosphorylase I.

The molecular weight of phosphorylase I was estimated by gel-filtration to be 204 000 ± 4000. The enzyme (form I) obtained after a further step of $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography on DEAE-cellulose appeared homogeneous on polyacrylamide gel electrophoresis. No phosphate was liberated when starch was omitted from the assay mixture, thus indicating that the enzyme preparation lacked phosphatase activity. The subunit molecular weight of this enzyme (phosphorylase I) was found to be about 95 000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Phosphorylase I is thus a dimeric protein as has been found with the enzymes from other plant sources [12].

Effect of pH

The pH optimum for the activity of phosphorylase I in 0.1 M citric acid/sodium citrate buffer was at pH 5.5—5.6, whereas that for phosphorylase II in 0.1 M sodium cacodylate/HCl buffer was at pH 6.2—6.3. These values are very close to those found with the corresponding isoenzymes of maize endosperm [7].

Effect of cations and other compounds

The effects of a number of substances on the activity of phosphorylase I are summarized in Table I. The enzyme was stimulated by Mg^{2+}, Mn^{2+}, AMP, adenosine 3:5′-cyclic monophosphate (cyclic AMP), pyruvate and EDTA (ethylenediamine tetra-acetate), but was inhibited by the other substances tested. Most of these effects are similar to those observed with phosphorylase I of maize endosperm [7]. Thus starch phosphorylase I of *V. subterranea*, like the corresponding enzymes from maize endosperm and potato, is active in the absence of AMP [3,7,8], but, unlike these other enzymes, is stimulated in the presence of AMP.

The percentage stimulation by AMP increased not only with increase in AMP concentration but also with increase in glucose-1-phosphate concentration; 4 mM AMP caused 37.5 and 51.0% stimulation in the presence of 2 mM and 8 mM glucose-1-phosphate respectively. Fig. 3a shows plots of initial velocity data at high glucose-1-phosphate concentrations as $1/v$ vs. 1/[glucose-1-phosphate] in the absence and presence of AMP. Plots of the data as $1/(v - v_0)$ vs. 1/[glucose-1-phosphate], where v_0 and v are velocities in the absence and presence of AMP respectively, are shown in Fig. 3b. Fig. 4a shows plots of initial velocity data as $1/v$ vs. 1/[AMP]. The plots are biphasic but appear parallel in the presence of low AMP concentrations (<1.3 mM) whereas plots of the data as $1/(v - v_0)$ vs. 1/[AMP] are linear and intersecting (Fig. 4b).

With muscle glycogen phosphorylase that has been desensitized by limited tryptic attack, or reaction with sulphydryl reagents or glutaraldehyde, inter-

TABLE I

EFFECTS OF CATIONS AND SOME OTHER SUBSTANCES ON THE ACTIVITY OF PHOSPHORYLASE I

The concentration of glucose-1-phosphate in the assay mixtures was 2 mM and that of each additive was 1 mM.

Substance added (1 mM)	Enzyme activity (μg/Pi liberated/30 min)	Inhibition (%)	Activation (%)
None	8.5	—	—
$MgSO_4$	13.5	—	58.8
$MnCl_2$	9.8	—	15.3
$FeSO_4$	7.8	8.2	—
$CuSO_4$	8.2	3.5	—
$ZnCl_2$	6.2	27.1	—
AMP	9.9	—	16.5
Cyclic AMP	10.5	—	23.5
ATP	6.3	25.8	—
Pyruvate	12.0	—	41.2
EDTA	18.2	—	114.1

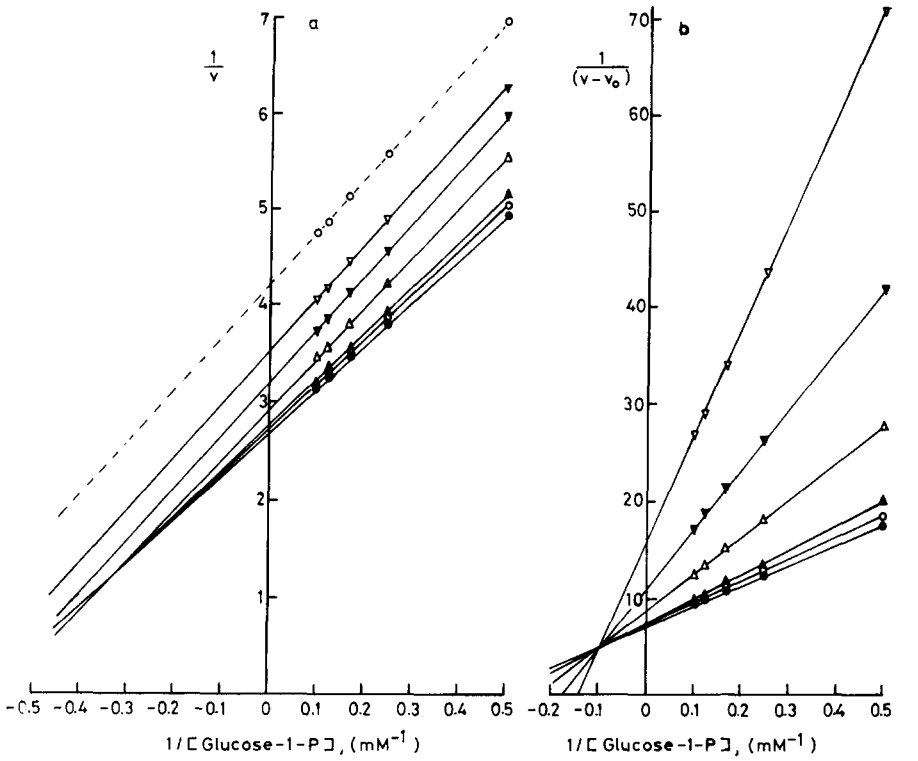

Fig. 3. Plots of initial velocity data of starch phosphorylase I. a, as $1/v$ vs. 1/[glucose-1-phosphate]; b, as $1/(v-v_0)$ vs. 1/[glucose-1-phosphate] in the absence (o- - - - -o) and presence of 0.5 mM (▽), 1.0 mM (▼), 2.0 mM (△), 3.0 mM (▲), 4.0 mM (○) and 5.0 mM AMP (●). v and v_0 are velocities (μg inorganic phosphate liberated/min) in the presence and absence of AMP respectively.

secting patterns (cf. Figs. 3b and 4b) are interpreted as showing that the affinity of the enzyme for glucose-1-phosphate is enhanced by the binding of AMP and that for AMP is similarly enhanced by the binding of glucose-1-phosphate [19]. A similar interpretation of the results shown in Figs. 3b and 4b suggests that the apparent K_m for glucose-1-phosphate decreases from 7.14 mM in the presence of 0.5 mM AMP to 2.78 mM in the presence of 5.0 mM AMP, and that for AMP decreases from 2.70 mM in the presence of 2 mM glucose-1-phosphate to 1.11 mM in the presence of 10 mM glucose-1-phosphate.

Double-reciprocal plots ($1/v$ vs. 1/[glucose-1-phosphate]) of initial velocity data obtained at low glucose-1-phosphate concentrations were non-linear (convex up) in the absence of AMP but linear in the presence of high concentration of AMP. These results indicate that AMP binds to a regulatory site in competition with glucose-1-phosphate functioning as an activator, and that when the regulatory site is saturated with AMP (or glucose-1-phosphate) double-reciprocal plots become linear. A similar effect was observed with ATP although it is an inhibitor.

Starch phosphorylase I from *V. subterranea*, like those from potato and maize endosperm, does not show the usual homotropic cooperativity between AMP or glucose-1-phosphate sites that is found with animal glycogen phos-

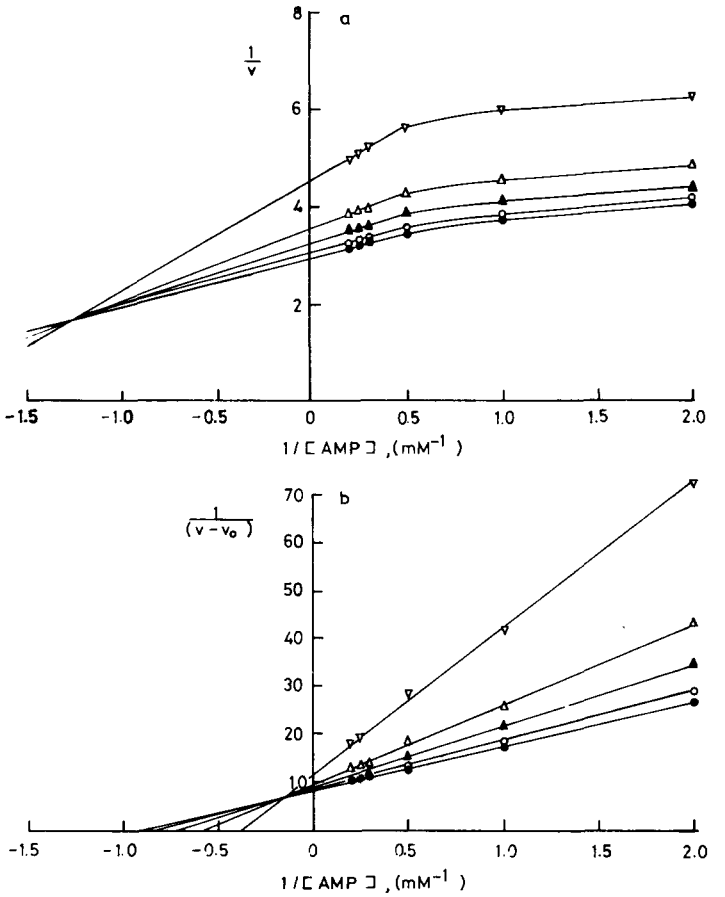

Fig. 4. Plots of initial velocity data of starch phosphorylase I. a, as $1/v$ vs. 1/[AMP]; b, as $1/(v-v_0)$ vs. 1/[AMP] in the presence of 2 mM (▽), 4 mM (△), 6 mM (▲), 8 mM (○) and 10 mM glucose-1-phosphate (●). v and v_0 are velocities (μg inorganic phosphate liberated/min) in the presence and absence of AMP respectively.

phorylase, although potato starch phosphorylase is similar to muscle glycogen phosphorylase in many respects [cf. Ref. 20]. Desensitized glycogen phosphorylase no longer shows homotropic cooperativity but still requires AMP for activity [19]. According to the sequential model of Koshland et al. [21], heterotropic interactions generated through ligand-induced conformational changes may or may not give rise to homotropic cooperativity, depending on the strength of interaction between subunits. Starch phosphorylase I from *V. subterranea* differs, however, from desensitized glycogen phosphorylase in that it is active in the absence of AMP, apparently with or without an activating molecule of glycose-1-phosphate occupying the regulatory site. It has been observed that in the absence of AMP high concentrations of inorganic phosphate activate muscle glycogen phosphorylase b [22], and that liver glycogen phosphorylase b is active in the absence of AMP contrary to general belief [23,24]. The properties of starch phosphorylase I from *V. subterranea* can thus be explained by these observations.

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