Amylopectin Degradation in Pea Chloroplast Extracts

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CAROLYN LEVI AND JACK PREISS
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Phosphorolysis rather than phosphorylation of amyolysis products was found to be the major pathway of sugar phosphate formation from amylopectin by pea (Pisum sativum L.) chloroplast stromal proteins. The $K_m$ for inorganic phosphate incorporation was 2.5 mM, and ATP did not stimulate amylopectin-dependent phosphate incorporation. Arsenate (10 mM) inhibited phosphate incorporation into glucose monophosphates up to 46% and phosphoglucomutase activity 96%, resulting in glucose 1-phosphate accumulation as a product of amylopectin degradation. The intracellular distribution of enzymes of starch utilization was determined. Phosphorylase, phosphoglucomutase, and hexokinase were found in the chloroplast and cytoplasm, while $\beta$-amylase was restricted to the cytoplasm. Maltase was not detectable; maltose phosphorylase was active in the chloroplast.

Chloroplasts must synthesize sugar phosphates which are transportable by the chloroplast inner membrane in order for photosynthetic products to be utilized by the cytoplasm. Previous studies have shown that starch, a major photosynthetic product, can be metabolized within the chloroplasts to glyceraldehyde-3-P (5, 8). Although further studies have shown that chloroplast starch degradation is accelerated by Pi (5, 17), it has not been clearly shown whether the phosphorylated intermediates originate entirely through phosphorolysis of starch to glucose-1-P or instead from free glucose, produced by successive hydrolyses, that is phosphorylated to glucose-6-P by hexokinase. Although maltose is also a major product of starch degradation (8), it is not clear whether chloroplast maltose arises through amyolysis or through maltose phosphorylase as suggested by Linden et al. (10).

This paper distinguishes between these two pathways in the chloroplast by using unfractionated chloroplast stromal proteins since the problem of membrane impermeability to amylopectin and to starch granules could be avoided. The distribution of enzymes unique to each pathway is also reported.

MATERIALS AND METHODS

Plant Material. Peas (Pisum sativum L. var. Progress No. 9) were shaded with a single layer of cheesecloth and growth chamber-grown under 12-hr, 24 C days and 12-hr, 18 C nights.

Preparation of Chloroplasts. Whole chloroplasts were prepared from 20- to 26-day-old pea shoots. Pea shoots (50-100 g) were homogenized for 1 sec in a VirTis 45 homogenizer in 200 ml of chilled isolation medium containing 0.33 M sorbitol, 10 mM Na-pyrophosphate, 50 mM HEPES buffer (pH 6.8), 5 mM MgCl$_2$, and 10 mM freshly prepared Na-ascorbate. The homogenate was filtered through single layers of cheesecloth and Miracloth and centrifuged 1.5 min at 2,500g. The pellets were washed with a total of 20 ml of a solution containing 0.33 M sorbitol, 50 mM HEPES (pH 7.8), 2 mM EDTA, 1 mM MgCl$_2$, and 1 mM MnCl$_2$ by gentle agitation of each pellet in 5-ml wash buffer, followed by centrifugation for 45 sec at 2,500g.

Preparation of Chloroplast Stromal Extract. Pellets of whole chloroplasts were broken osmotically with a cotton-tipped rod by suspension in 10 to 20 ml of 10 mM HEPES (pH 6.8) containing 2.5 mM dithioerythritol. The broken chloroplasts were centrifuged at 30,000g for 15 min and the supernatant fluid used for assays. For $^{32}$Pi fixation assays, the chloroplasts were broken in the above wash solution diluted 1:25, containing 1 mM dithioerythritol, 5 mM MgCl$_2$, and 5 mM NaHCO$_3$ and the supernatant fluid obtained after centrifugation was concentrated to 2 to 3 ml in an Amicon concentrator with a P-10 membrane.

Preparation of Supernatant Extract for Enzyme Assays. A 10- to 25-ml portion of the combined supernatants obtained after centrifugation of the intact chloroplasts was brought to 2.5 mM dithioerythritol and centrifuged at 30,000g for 15 min. The resulting supernatant was used for assays.

Reagents. $^{32}$P-labeled phosphate was obtained from New England Nuclear and used after acid hydrolysis. P-glucomutase (rabbit muscle), triose-P isomerase and aldolase were obtained from Boeringer Mannheim. Glucose-6-P dehydrogenase, hexokinase, $\alpha$-glycerophosphate dehydrogenase and lactate dehydrogenase were obtained from Sigma Chemical Co. C. Boyer, University of California, Davis, prepared the maize starch granules. Other reagents were obtained at the highest purity from commercial sources.

$^{32}$Pi Fixation Assay. Bound $^{32}$P was assayed by the method of Avron (1).

Assay of Intermediates. Total reducing sugars were measured by the method of Nelson (14) and protein by the method of Lowry et al. (11).

Glucose was assayed by following the reduction of NADP at 340 nm using reaction mixture containing 50 $\mu$mol of MgCl$_2$, 0.23 $\mu$mol of ATP, 0.46 units of glucose-6-P dehydrogenase, 0.46 units of hexokinase, and 0.1 to 0.2 ml of sample in 1 ml total volume. Glucose-6-P and glucose-1-P were assayed in 1 ml with 50 $\mu$mol Tris-Cl (pH 7.5), 0.10 mg of BSA, 0.4 mg of NADP, 1 $\mu$mol of MgCl$_2$, 65 $\mu$mol of glucose-1,6-P$_2$, 0.46 units of glucose-6-P dehydrogenase, and 5 $\mu$g of P-glucomutase. A unit is defined as that amount of enzyme catalyzing the conversion of 1 $\mu$mol of substrate into product in 1 min.

ENZYME ASSAYS

Assay of P-Glucomutase in Pea Extracts. The reaction mixtures contained 50 $\mu$mol of HEPES (pH 6.8), 0.1 mg of BSA, 0.2 mg of NADP, 0.02 $\mu$mol of glucose-1,6-P$_2$, 2 $\mu$mol of MgCl$_2$, 0.9 unit of glucose-6-P dehydrogenase, 2 $\mu$mol of glucose-1-P, and 1 ml total volume. Activity was
followed by reduction of NADP at 340 nm. Commercial P-glucomutase was assayed by the same procedure except that 0.45 units of glucose-6-P dehydrogenase and 65 nmol of glucose-1,6-P₂ were used.

**Assay of ADP-Glucose Pyrophosphorylase.** ADP-glucose pyrophosphorylase was assayed in the pyrophosphorylase direction as described by Leveni and Preiss (9).

**Assay of Hexokinase.** The reaction mixtures contained 50 μmol of HEPES (pH 7.8), 0.1 mg of BSA, 0.2 mg of Na-phosphate (pH 7.4), 0.2 mg of NADP, 1 μmol of ATP, 50 μmol of glucose, and 0.9 unit of glucose-6-P dehydrogenase, and sample in a total volume of 1 ml. Activity was followed by reduction of NADP at 340 nm.

**Assay of Phosphorylase.** The reaction mixtures contained 50 μmol of HEPES (pH 6.8), 0.1 mg of BSA, 10 μmol of Na-phosphate (pH 7.4), 0.2 mg of NADP, 1 mg of amylpectin, 0.02 μmol of glucose-1,6-P₂, 55 μg of P-glucomutase, 0.46 unit of glucose-6-P dehydrogenase, and sample in 1 ml total volume. Activity was followed by reduction of NADP at 340 nm.

**Assay of β-Amylase.** The reaction mixtures contained 50 μmol of HEPES (pH 6.8), 1 mg of amylpectin, and sample to 1 ml total volume. The reactions were terminated after incubation (30–60 min) at 26°C in boiling water for 1 min and centrifuged in a clinical centrifuge to remove precipitated protein. Total reducing sugars were assayed by the method of Nelson (14).

**Assay of Phosphorfructokinase.** The reaction mixtures contained 50 μmol of HEPES (pH 6.8), 2 μmol of MgCl₂, 2.5 μmol of dithioerythritol, 1.5 μmol of fructose-6-P, 0.5 μmol of ATP, 0.1 mg of NADH, 0.01 mg of tris-P-isomerase, 0.9 unit of α-glycero-P dehydrogenase, 0.8 unit of aldolase, and sample to 1 ml total volume. Activity was followed by oxidation of NADH at 340 nm.

**Assay of Pyruvate Kinase.** The reaction mixture contained 50 μmol of HEPES buffer (pH 6.8), 2 μmol of P-enolpyruvate, 2.2 μmol of ADP, 50 μmol of KCl, 10 μmol of MgCl₂, 0.15 mg of NADH, 2.5 μmol of dithioerythritol, 7 units of lactate dehydrogenase, and sample to 1 ml total volume. Activity was followed by oxidation of NADH at 340 nm and corrected for activity in the absence of ADP.

**Assay of Maltase.** The reaction mixtures contained 50 μmol of Na-acetate buffer (pH 6), 100 μmol of maltose, and sample to 1 ml total volume. The reactions were terminated after 10-min incubation at 26°C by 2-min boiling, clarified by centrifugation, and assayed for glucose as described previously.

**RESULTS**

**Amylopectin Degradation.** The apparent Km for 32Pi fixation by chloroplast stroma was 2.4 mm and fixation saturated at 6 mm (Fig. 1). No 32Pi was incorporated in the absence of added amylpectin. ATP stimulated 32Pi fixation (Table I), but ATP stimulation was not dependent upon added amylpectin.

Table II shows that free glucose was present in the reaction mixtures, but glucose was not a product of amylpectin degradation unless arsenate was added to the reaction mixture. The total concentration of glucose was lower when ATP was added, indicating active hexokinase (see Table IV).

The ratio of glucose-1-P to glucose-6-P as products of amylpectin degradation (Table II) in the absence of arsenate was at or close to equilibrium value of about 0.06 (13) (Table II). Arsenate caused a strong shift in favor of glucose-1-P. This is due to inhibition of P-glucomutase by arsenate (7).

Table III shows the effect of 10 mM arsenate on a commercial preparation of P-glucomutase and on P-glucomutase activity in pea chloroplast stroma. Incubation of the reaction mixtures with added glucose-1,6-P₂ prevented inhibition.

No glucose, glucose-6-P, or glucose-1-P was formed when 10 mM arsenate was added to the reaction mixtures. Glucose-1-P was formed when 10 mM arsenate was added in the presence of ATP and ADP, but was not formed in their absence.

**Enzyme Distribution.** The distributions of enzymes of starch synthesis and carbohydrate utilization between pea shoot chloroplasts and supernatant fractions are compared in Table IV. Glucose-1-P was formed when 10 mM arsenate was added to the reaction mixtures. Glucose-1-P was formed when 10 mM arsenate was added in the presence of ATP and ADP, but was not formed in their absence.
This resulted of phosphorolysis by $^{32}$Pi rather of preparation and range present in higher results starch phosphorylase marker, observations chloroplast fraction. present in both fractions, $\beta$-amylase and the cytoplasmic marker, pyruvate kinase (15), were primarily in the supernatant, while ADP-glucose pyrophosphorylase was restricted to the chloroplast fraction.

Evidence that $\beta$-amylase and not $\alpha$-amylase was the amylase assayed is that heat treatment for 5 min at 70 C in the presence of 20 mM CaCl$_2$ completely abolished activity, whereas 12.5 mM EDTA had no effect on activity. This is consistent with the observations of Swain and Dekker (18).

No maltase activity could be detected in either fraction. Maltose phosphorylase (16) was present in the chloroplast fraction (Table IV).

**DISCUSSION**

Stoop et al. (17) have shown that up to 2.5 mM Pi accelerates starch degradation in isolated, intact spinach chloroplasts. They interpreted this as evidence for phosphorolytic degradation. The results shown in Figure 1 are consistent with this interpretation. The higher Pi requirement (6 mM for saturation) in the extract than seen in intact plastids (17) is probably due to endogenous Pi present in the intact plastids which was diluted out in preparation of the extract. Indeed, the $K_m$ of 2.4 mM and saturation at 6 mM seen in pea chloroplast extract was within the range of known $K_mE$ values for plant phosphorylases (2, 4, 6) and much higher than the $K_mE$ for Pi of triose-P dehydrogenase (19), the other entry point of free Pi into glycolysis. ATP stimulation of 32Pi fixation (Table I) may be due to triose-P dehydrogenase and P-glyceraldehyde kinase-catalyzed exchange of 32Pi into ATP.

Phosphorylase catalyzed arselenolysis of amyllopectin in the presence of 10 mM arsenate (Tables I and II). However, inhibition of P-glucomeatal activity was much greater than inhibition of phosphorolysis of amyllopectin (Tables II and III). This resulted in the accumulation of the glucose-1-P produced by phosphorylase of amyllopectin. Since glucose-1-P accumulated rather than glucose-6-P it is evident that phosphorylase rather than hexokinase is the main source of sugar phosphate synthesis from amylopectin.

Table IV shows the intracellular distribution of phosphorylase and $\beta$-amylase. These data plus the observation showing the presence of maltose phosphorylase and absence of maltase in the chloroplast fraction are consistent with a phosphorolytic mechanism of chloroplast starch degradation.

Since maltose is a product of photosynthesis (10) and of starch degradation in intact chloroplasts (5, 8), the low level of maltase in chloroplasts may mean that maltose phosphorylase is a mechanism for degradation as well as synthesis of maltose in chloroplasts.

Swain and Dekker (18) have proposed that pea shoot $\beta$-amylase functions to degrade oligosaccharides moving through the vascular system from the seed into the shoot. They also noted that the level of maltase activity in the shoot is very low. The finding of $\beta$-amylase predominantly in the cytoplasm rather than in the chloroplast is consistent with this view (cf. ref. 3 and Table IV).

Finally, $\alpha$-amylase is not found in pea shoots (18), and our data show that $\beta$-amylase and maltase do not contribute to glucose formation in pea chloroplasts. Amylopectin in pea chloroplasts is therefore mainly degraded via the phosphorylase reaction. This is in contrast to what is commonly believed to be the main degradative route for storage tissue starch where $\alpha$-amylase and maltase are the primary degradative enzymes (12).

**LITERATURE CITED**

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