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ENZYMIC DEGRADATION OF STARCH IN COTTON LEAVES

CHONG W. CHANG

USDA, Science and Education Administration—Agricultural Research, Western Cotton Research Laboratory, 4135 E. Broadway Road, Phoenix, AZ 85040, U.S.A.

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton pathways; starch breakdown; phosphorylase; phosphoglucomutase; hexokinase; alpha-amylase; glucose; maltose; glucose-1-P; glucose-6-P.

Abstract—Starch degradation enzymes and their products were determined from chloroplasts and leaves of cotton (*Gossypium hirsutum* L. cv Coker 100) exposed to a 14-hr dark period to clarify pathways of starch degradation. In chloroplasts, phosphorylase and phosphoglucomutase were in soluble forms. Hexokinase, however, was rather firmly associated ionically with starch granules. In contrast, the majority of alpha-amylase bound to the starch granules strongly resisted the ionic solvent extraction. The magnitudes of the enzyme activities of bound alpha-amylase and hexokinase were 4.5–7-fold higher than those of phosphorylase and phosphoglucomutase. The levels of glucose and maltose degraded from starch granules were significantly greater than those of glucose-1-P and glucose-6-P. The initial starch granule hydrolysis occurred solely by the action of alpha-amylase. Phosphorylase, however, depended on the amylolytic degradation products for phosphorolysis. In leaves, the levels of bound alpha-amylase and hexokinase were 2–4-fold higher than those of phosphorylase and phosphoglucomutase throughout the entire dark period. Alpha-amylase and hexokinase activities increased with darkness and reached a maximum at about 10 hr. Phosphorylase (accompanied by a negligible level of phosphoglucomutase) rose during the period when alpha-amylase activity increased rapidly and reached a maximum following the peak activity of alpha-amylase. It was concluded that starch granules in chloroplasts are hydrolyzed initially by the sole action of bound alpha-amylase and the flow of degradation products is highly restricted to the pathway involving alpha-amylase and hexokinase.

INTRODUCTION

The conversion of starch to soluble sugars could be phosphorolytic, involving alpha-glucan phosphorylase (EC 2.4.1.1) and phosphoglucomutase, (EC 2.7.5.1) or hydrolytic, entailing amylases (alpha-amylase, EC 3.2.1.1 and/or beta-amylase, EC 3.2.1.2) and hexokinase (EC 2.7.1.1), or the conversion could be due to some combination of phosphorolysis and hydrolysis [1]. Stitt *et al.* [1] reported the phosphorolytic pathway of starch depletion from pea leaf chloroplasts, which contained more phosphorylase than amylase activity. Levi and Preiss [2] proposed a similar pathway of amylopectin degradation from pea shoot chloroplast extracts. Pea leaves contained both alpha-amylase and phosphorylase [1]. Previous studies [1, 2], however, did not clarify how starch granules were initially degraded by the actions of these two types of enzymes in pea. Levi and Gibbs [3] found that starch was degraded within spinach chloroplasts to glyceric acid 3-phosphate and to maltose, indicating a system involving both phosphorolysis and hydrolysis. Peavey *et al.* [4] determined degradation products after incubating chloroplasts containing ^{14}C-labeled starch prepared from spinach. They reported that chloroplastic starch was degraded principally by the Embden–Meyerhoff pathway and by a pathway involving amylolytic cleavage. With similar experimental materials, Pongratz and Beck [5] found that the amylolytic pathway of starch breakdown exhibited a distinctive diurnal oscillation as compared with the phosphorolytic pathway. These studies, however, did not provide much information on the roles of the initial enzymic starch granule solubilization in chloroplasts.

Starch accumulates in chloroplasts by a growth of starch granules [6]. Cotton contained 10–24 mg of starch/g fresh leaves [7]. The actions of hydrolytic and phosphorolytic enzymes on starch granules in cotton were unknown. Therefore, the localization of starch metabolising enzymes in chloroplasts and the sequential enzymic reactions were studied for a better understanding of starch degradation pathways in cotton leaves.

The purposes of the present study were to locate starch metabolising enzymes within cotton chloroplasts and to clarify the dependence of phosphorolytic degradation of starch metabolites on the initial amylolysis of starch granules. The predominant amylolytic pathway over phosphorolysis in intact leaves was also investigated during an extended dark period.

RESULTS AND DISCUSSION

As shown in Fig. 1, both phosphorylase and

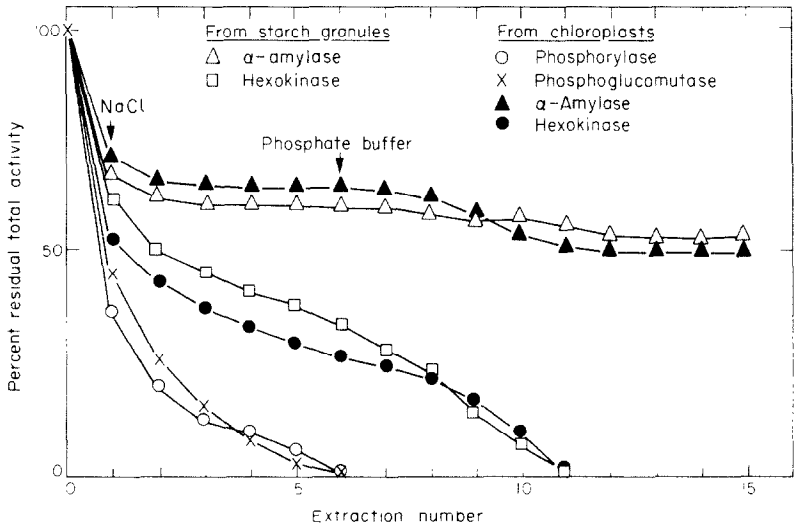

Fig. 1. Extractibilities of phosphorylase, phosphoglucomutase, alpha-amylase, and hexokinase from cotton chloroplasts, and those of alpha-amylase and hexokinase from starch granules. Chloroplasts and starch granules were isolated from 55 g of fresh cotton leaves and assayed for enzyme activities as described in the Experimental. Chloroplasts (equivalent to 1.32 mg chlorophyll) or starch granules (36.6 mg starch) were successively extracted for enzymes with 1 M NaCl and then 0.2 M Na-phosphate buffer at pH 7.6. The total activity of each enzyme associated with chloroplasts or starch granules prior to extraction was assigned a value of 100%. These values are shown in the text.

phosphoglucomutase were readily released from isolated chloroplasts by succesive extractions with NaCl solution. Hexokinase, however, resisted extractions with salt and buffer at pH 7.6. The complete release of this enzyme from chloroplasts required more extractions than phosphorylase and phosphoglucomutase. In contrast, extensive extraction of alpha-amylase with similar extraction media resulted in only about 40% enzyme release from the total activity. The remaining activity was tightly bound to the residue. Since the broken chloroplast pellets were composed mainly of starch granules and chloroplast membranes, the enzyme association with these components was further investigated. Extractability of hexokinase and alpha-amylase from isolated starch granules was similar to that of the two enzymes from intact chloroplasts. None of the four enzymes tested banded with chloroplast membranes in the sucrose at a mean density of 1.12 g/cm^3, because the first membrane extract contained negligible levels of enzyme activities. These data show that phosphorylase and phosphoglucomutase are in soluble forms. Hexokinase, however, is rather firmly bound ionically to starch granules. In contrast, about 65% of the alpha-amylase in chloroplasts was tightly associated with starch granules.

Total enzyme activities (μmol $\times 10^{-3}$/min) of chloroplasts (equivalent to 1.32 mg chlorophyll) prior to extractions were 8.3 Pi, 2.2 NADPH, 36.3 glucose equivalent, and 14.6 NADH for phosphorylase, phosphoglucomutase, alpha-amylase, and hexokinase, respectively. Therefore, the activities of the two bound enzymes responsible for hydrolysis are predominant over those for phosphorolysis.

As shown in Fig. 2, increasing soluble alpha-amylase produced increasing degradation products from starch granules. The alpha-amylolytic attack on cotton starch granules also was observed when starch granules bound with alpha-amylase *in vivo* (1 M NaCl-washed starch granules) were incubated in the presence of orthophosphate (insert). In these experiments, the production of glucose during the digestion of starch granules by the action of alpha-amylase was directly proportional to the total reducing power generated. Paper chromatographic separation of the species of degradation products from cotton starch granules catalyzed by its own bound alpha-amylase were glucose, maltose, and glucose oligomers. Identification of the tightly bound *in vivo* alpha-amylase (Fig. 1) which is capable of attacking starch granules has not been previously reported.

As shown in Fig. 3, phosphorylase activity increased in the presence of constant amounts of starch granules with rising alpha-amylase. Increasing alpha-amylase without starch granules, however, did not contribute to increasing phosphorylase activity (see constant blank value). Phosphorylase alone also failed to degrade starch granules (insert). Since alpha-amylase produced various degradation products including glucose oligomers from starch granules, phosphorylase activity depended on alpha-amylase activity, which released substrates from starch granules for phosphorylase.

As shown in Fig. 4, both phosphorylase activity (A) and alpha-amylase activity bound to starch granules (B) were decreased by EDTA. EDTA inhibits alpha-amylase [8], but does not influence phosphorylase activity [8]. Increasing EDTA without alpha-amylase bound starch granules did not contribute to decreasing phosphorylase and alpha-amylase activities (see constant blank value) (C). The data show that phosphorylase activity depends on the

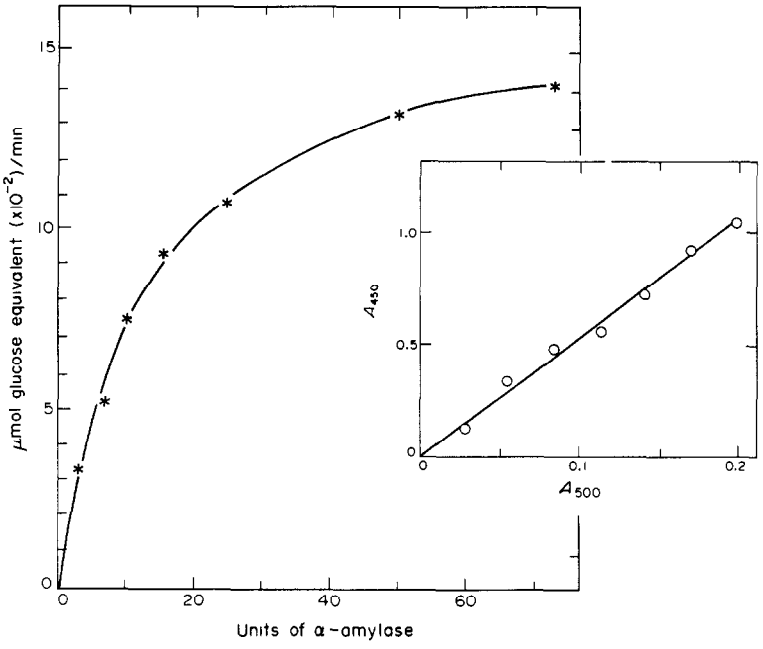

Fig. 2. The action of alpha-amylase activity on cotton starch granules. The reaction mixture contained 5 mg of toluene-washed starch granules, 4.08 mmol of KH_2PO_4 (pH 4.5), one drop of 0.2 M $CaCl_2$ and various units of purified soluble cotton alpha-amylase in 1.5 ml total volume. The mixture was incubated at 30° for 60 min. Reducing sugar produced was estimated by the Nelson–Somogi method [30]. Insert: digest conditions were similar to those for above experiments except that NaCl-washed starch granules were substituted for toluene-washed starch granules including alpha-amylase, and samplings were made at various time intervals. Glucose and reducing power released were estimated by reading samples at A_{450} [32] and A_{500} [30], respectively.

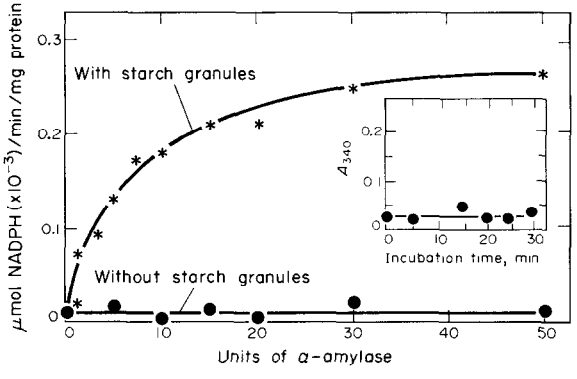

Fig. 3. Dependence of phosphorylase activity on alpha-amylase action on starch granules. Phosphorylase activity was assayed by the procedures of Levi and Preiss [2] except for the following modifications. The reaction mixture contained 5 mg toluene-washed starch granules, 8 units of purified phosphorylase, one drop of 0.2 M $CaCl_2$, and various units of alpha-amylase instead of amylopectin in 1.2 ml total volume. The mixture was incubated at 30° for 10 min by shaking. The reaction was terminated by boiling for 25 sec. The clear supernatant after mixture centrifugation was read at 340 nm. The short boiling process did not change A_{340} during the first 5 min, but decreased NADPH by about 5%. Insert: assay conditions were similar to above experiments except that no alpha-amylase was included in the reaction mixture and samplings were made at various time intervals.

action of alpha-amylase bound to starch granules *in vivo*.

As shown in Fig. 5. about 75% of the total leaf starch content decreased rapidly during the 14-hr dark period (top). Relative to this, bound alpha-amylase activity showed a sharp increase almost immediately after the onset of darkness. The activity reached a maximum after 10 hr of darkness and thereafter declined (the maximum soluble alpha-amylase activity was 0.86×10^{-3} μmol glucose equivalent/min/mg protein, which was relatively too low to be graphed). In contrast, soluble phosphorylase activity decreased during the first 4 hr period. The activity, however, began to rise when the rate of increase in alpha-amylase activity was fastest. The maximum activity of soluble phosphorylase was attained later than that of alpha-amylase. Bound alpha-amylase activity was 2–4 fold higher than soluble phosphorylase throughout the entire dark period. These data show that in the dark, starch is degraded initially by bound alpha-amylase. Thereafter, further degradation occurs by the actions of both alpha-amylase and phosphorylase, with the former enzyme activity being much higher than the latter enzyme. Also, the course of change in phosphorylase activity appears to be influenced by alpha-amylase. This relationship in intact leaves is compatible with the results shown in Figs. 3 and 4.

As shown in Fig. 5 (bottom), the level of starch-granule bound hexokinase activity was more than 4-

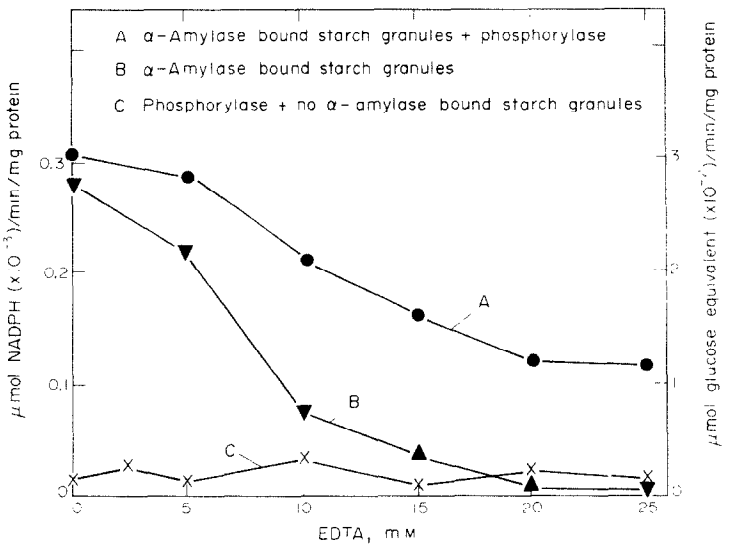

Fig. 4. Dependence of phosphorylase activity on alpha-amylase activity influenced by EDTA. Assay conditions for phosphorylase (A) were similar to those for Fig. 3 except that NaCl-washed starch granules and EDTA were substituted for toluene-washed starch granules and alpha-amylase, respectively. Assay conditions for alpha-amylase (B) were similar to those for Fig. 2 except that NaCl-washed starch granules and EDTA were substituted for toluene-washed starch granules and alpha-amylase, respectively. The assay conditions for experiments (C) were similar to those for (A) except that no alpha-amylase-bound starch granules were included in the reaction mixtures.

fold greater than the magnitudes of the soluble hexokinase and phosphoglucomutase activities, which were practically negligible. The pattern of change in bound hexokinase activity was similar to that of bound alpha-amylase (Fig. 5, top) except that hexokinase began to increase only after an initial approximately 4 hr lag period. The greater magnitudes of bound alpha-amylase and hexokinase as compared with those of soluble phosphorylase and phosphoglucomutase in intact leaves seemed to reflect those enzymes in chloroplasts (the second paragraph under Fig. 1, Results and Discussion).

As shown in Table 1, the ratio of glucose-6-P to glucose-1-P as degradation products of starch granules bound with alpha-amylase in the presence of orthophosphate was at, or close to, an equilibrium value of about 16.7 [2]. The levels of glucose and maltose degraded from starch granules were significantly greater than those of glucose-1-P and glucose-6-P in the presence or absence of orthophosphate. The data were related to the levels of alpha-amylase and phosphorylase in chloroplasts (the second paragraph under Fig. 1, Results and Discussion) and in intact leaves (Fig. 5, top).

Responses of purified alpha-amylase, hexokinase and phosphorylase to filtrates free of protein (MW 100 000) prepared from leaves showing various levels of enzyme activities were uniformly constant. The results indicated that enhanced activities of these enzymes (Fig. 5) were not due to the influence of small MW, cellular compounds. Therefore an allosteric influence of cellular metabolites on these enzymes was ruled out.

Also, during the 14 hr dark period, protein content decreased slightly during the first 2 hr dark period and then began to rise gradually to a maximum in the next 8 hr. Thereafter the protein content decreased. This pattern of change in the dark was similar to that of protein during the 10 hr light period indicating a possible relationship of an endogenous diurnal oscillation of protein content with the enhanced enzyme activities (Fig. 5). The increased levels of enzyme activities in the dark were also reported by others, but were attributed to various causes [5, 9].

Additional enzymes possibly associated with starch degradation in cotton were examined. Alpha-glucosidase (EC 3.2.1.20) [10] was present in chloroplasts, but was not bound to starch granules and did not degrade starch granules. Beta-amylase [10] and limit-dextrinase (EC 3.2.1.33) [11] were not found in cotton chloroplasts nor with starch granules. Therefore, alpha-amylase and hexokinase (Fig. 1) were the only starch metabolising hydrolytic enzymes which were bound to starch granules. These enzymes seemed to be of starch-granule origin, since, in addition to the supporting data in Fig. 1, the cytosolic marker glyceraldehyde-3-dehydrogenase (EC 1.2.1.12) and mitochondrial marker citrate synthase (EC 4.1.3.28) [12] were not detected with starch granules washed once. Also maltose phosphorylase (EC 2.4.1.8) [2] was not bound to starch granules, but was present in chloroplasts. The level of this enzyme, however, was extremely low as compared with maltase in cotton. Therefore, the source of glucose from glucose-1-P was assumed to be negligible, if any. The derivation of glucose by glucose-6-phosphatase was unlikely to occur since this enzyme has not been reported in chloroplast preparation [4]. The higher levels of

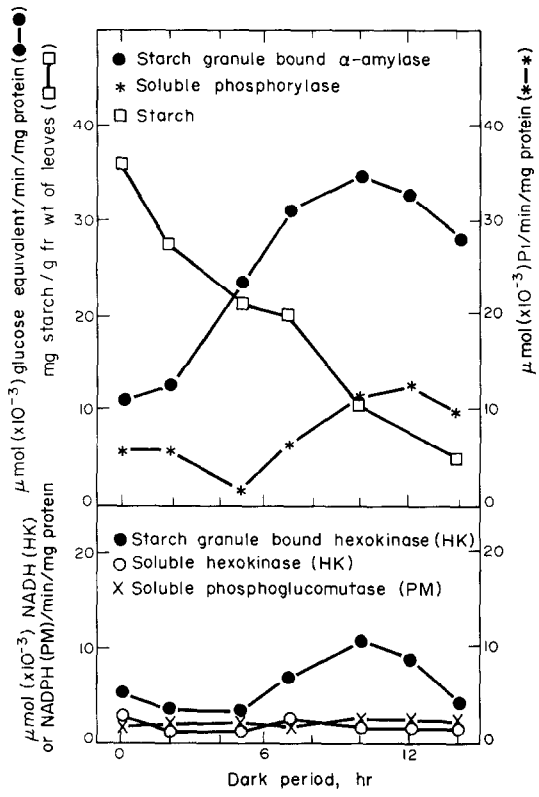

Fig. 5. Relationships of changes of phosphorylase, phosphoglucomutase, alpha-amylase, and hexokinase with starch degradation in cotton leaves during the 14-hr dark period. Fresh cotton leaves (10 g) were harvested at various dark periods from 35 day old plants. Soluble and bound enzymes were isolated and their activities were determined as described in the Experimental.

Table 1. Degradation of starch granules by cotton chloroplast extract

Addition	Glucose-1-P	Glucose-6-P	Glucose
	nmol/mg protein		
None	1.6 ± 0.06	21 ± 0.13	522 ± 4
15 μmol PO_4	3.8 ± 0.12	68 ± 1.8	491 ± 9*

Chloroplast suspension was prepared from 75 g of leaves and divided into two equal portions. Starch granules or concentrated enzyme proteins were prepared separately from each portion (see Experimental for procedures) and then combined. The combined suspension was used as the soluble chloroplast enzyme extract containing starch granules. The reaction mixture contained 50 μmol HEPES buffer (pH 6.8), 2 μmol $MgCl_2$, 0.5 ml starch granule suspension (1.9 mg protein), and additions as indicated in a total volume of 1.5 ml. The reaction was incubated for 60 min at 26° and stopped in a boiling water bath for 1 min. The samples were centrifuged and the supernatants were assayed for contents of intermediates by the enzymic procedure as described by Levi and Preiss [2].

*The glucose value was also compared with maltose content (321 nmol/mg protein) by analyzing intermediates in reaction mixtures by paper chromatography [31].

Each value was an average of three replicates and is shown ± s.e.

glucose and maltose as compared with glucose-1-P and glucose-6-P (Table 1) were most likely degradative products from starch.

The hydrolytic action of alpha-amylase was demonstrated not only by experiments with starch granules, to which soluble alpha-amylase was added (Figs. 2 and 3), but also by experiments involving native starch granules bound with enzymes *in vivo* (Fig. 2). In contrast, phosphorylase was not bound to starch granules in chloroplasts (Fig. 1) and was incapable of degrading the granular form of this polymer (insert, Fig. 3). This enzyme activity further depended upon the action of alpha-amylase, which initiated starch granule degradation (Fig. 4). This sequential relationship occurring between these two enzymes in chloroplasts was consistently reflected in the intact leaves exposed to an extended dark period (Fig. 5).

The magnitudes of bound alpha-amylase and hexokinase were much higher than those of phosphorylase and phosphoglucomutase in chloroplasts (the second paragraph under Fig. 1, Results and Discussion). Higher levels of these enzymes which are involved in the amylolytic pathway were maintained throughout the entire dark period in leaves (Fig. 5). Therefore the flow of starch degradation products (glucose and maltose) involved in hydrolysis is conspicuously greater than that of products (glucose-6-P and glucose-1-P) entailed in phosphorolysis (Table 1). At least part of glucose and maltose might be exported from chloroplasts via "glucose translocator" [13] in cotton rather than "phosphate translocator" for triose-P and P-glycerate known to function in spinach [3, 14].

It was concluded that starch granules in cotton chloroplasts are hydrolyzed initially by the sole action of an alpha-amylase bound to starch granules and the flow of degradation products is highly restricted to the pathway involving alpha-amylase and hexokinase. The fact of predominant amylolysis over phosphorolysis found in cotton strikingly contrasts with pea and spinach leaves, in which phosphorolysis is the main pathway of starch degradation [2, 1]. This study presents critical infomation on enzymic mechanisms associated with starch granule amylolysis occurring in cotton in comparison with other reports [1–5, 15–17] on starch degradation.

EXPERIMENTAL

Plant materials. Glandless cotton plants (*Gossypium hirsutum* L. cv Coker 100) were grown in a growth chamber as previously described [18].

Preparation of chloroplasts and enzyme extractions. Whole chloroplasts were isolated by the procedure of ref. [19], which was modified with media used in ref. [2]. The top 3 to 4 leaves of about 30-day-old plants were harvested after a 4-hr photoperiod. The light intensity was maintained rather low at a PAR of 426 μE/m^2/sec to minimize starch granules, which can cause chloroplast breakage. Leaves (about 100 g)

were cut into small pieces with scissors and sub-divided into about 4 equal small portions. Each portion was ground by hand in a mortar (without sand) with *ca* 1.5 vol of chilled grinding medium (0.33 M sorbitol, 10 mM Na-pyrophosphate, 50 mM HEPES buffer at pH 6.8, 5 mM $MgCl_2$, and 10 mM freshly prepared Na-ascorbate for phosphorylase, phosphoglucomutase and alpha-amylase; 0.4 M sucrose, 0.01 M NaCl, and 0.05 M Tris–HCl at pH 7.8 for hexokinase). The slurry from each grinding was combined and then squeezed through 4 layers of Miracloth. Chloroplasts were sedimented from the cell-free filtrate by 3 successive, brief, low-speed centrifugations (namely 200*g*, 325*g*, and 580*g*) as previously described [19]. The sedimented chloroplasts were washed with the same grinding medium by centrifugation for 30 sec at 2500*g* (the phase contrast of the chloroplast suspension in hypotonic conditions showed that 50 to 70% of the chloroplasts were highly reflecting, indicating the relative integrity of chloroplast envelopes). In some experiments, chloroplasts were isolated by the procedure of ref. [20], which produced about 71% chloroplast envelope integrity by the ferricyanide-dependent O_2 evolution method [21]. Pellets of whole chloroplasts were broken osmotically in extraction media, 1 M NaCl and 0.2 M Na phosphate at pH 7.6. Soluble and residual enzymes were then separated by successive extractions.

Isolation of chloroplast membranes. After repeated washings, the residual broken chloroplasts were suspended in 15 ml of breaking medium [2]. 5 ml portions of this suspension were layered onto 15 ml of a 30 to 60% (1.12 to 1.28 g/cm^3) sucrose density gradient with 10 ml of 65% (1.32 g/cm^3) sucrose for cushion in each of 3 centrifuge tubes (34 ml capacity). The tubes were spun at 1935*g* for 5 min and then at 12 100*g* for an additional 10 min in a Beckman SW 25.1 rotor. A dark green band was separated at the interface between water and the 30% sucrose gradient (1.12 g/cm^3), which was discrete from the density (1.19 g/cm^3) for cotton mitochondria [22]. The green membrane layer was pipetted out for enzyme assays after removing the top water layer. The qualitative test for starch by the Anthrone method [18] showed a negative result with this green material, which therefore was completely separated from starch granules.

Starch granule isolation. Starch granules were isolated as described previously [23].

Preparation of toluene- and NaCl-*washed starch granules.* Isolated starch granules [23] were repeatedly washed with toluene [24] or with 1 M NaCl. Starch granules treated with toluene contained no activities of phosphorylase and alpha-amylase. Materials washed with NaCl, however, were associated with a considerable amount of residual alpha-amylase, but with negligibile levels of phosphorylase activity as shown in Figure 1. Starch granules so treated were used for experiments in Figs. 2, 3, and 4.

Isolation of starch degrading enzymes from cotton leaves. 8 to 10 g of cotton leaves were homogenized in 55 ml of 0.3 M borate buffer at pH 7.6 containing 0.05 M Na ascorbate [25]. The homogenate was filtered through 3 layers of cheesecloth and centrifuged at 30 900*g* for 20 min. The supernatant was filtered through a sheet of Whatman No. 1 filter paper and the filtrate was centrifuged at 124 000 *g* for 60 min. 15 ml of the resulting supernatant was loaded onto a Bio-Gel P-60 column. The first 45 ml of eluate, which contained no protein, was discarded. The next 30 ml of eluate, which contained protein, was concd to *ca* 4 ml with an Amicon ultrafiltration cell using a PM-10 membrane. The concd protein was precipitated by bringing the soln to different % saturations of $(NH_4)_2SO_4$ for each enzyme (20–50% for phosphorylase, 0–50% for phosphoglucomutase, and 50–65% for soluble hexokinase). The mixture was centrifuged and the ppt was dissolved in *ca* 2 ml of H_2O. The sample was dialyzed against various buffer solns containing 0.3 ml/l. mercaptoethanol (phosphorylase with 0.01 M maleic acid–NaOH buffer at pH 6.2, phosphoglucomutase and soluble hexokinase with 0.02 M Tris–HCl at pH 7.5). Soluble alpha-amylase was isolated by the procedure of ref. [10] and further purified [26]. For bound alpha-amylase and hexokinase, enzymes bound to starch granules were isolated by the procedure of ref. [23] in order to see the compatibility with those in chloroplasts.

Enzyme assays. Activities of phosphorylase (in an excess of orthophosphate) [2], phosphorylase (in an excess of glucose-1-P) [27], phosphoglucomutase [28], and soluble hexokinase [29] were determined by the procedures of the refs cited. Starch-granule bound hexokinase was assayed for activity similarly to soluble hexokinase with minor modifications. The reaction mixture was shaken in a water for 10 min, boiled for 25 sec, and centrifuged. The clear supernatant was read at 340 nm. The short boiling did not change A_{340} during the first 5 min, but decreased NADH subsequently by *ca* 5%, which was accordingly corrected. Soluble alpha-amylase activity was determined as reported earlier [10]. For starch-granule bound alpha-amylase activity, the reaction mixture contained 0.34 mg soluble starch, 4.08 mmol of KH_2PO_4 (pH 4.5), enzyme bound starch granules equivalent to 0.25 mg protein, and one drop of 0.1 M $CaCl_2$ in 1 ml total vol. The mixture was shaken at 30° for 30 min. Reducing sugar produced was estimated by the method of ref. [30]. Enzyme activities of alpha-amylase bound to starch granules before and after heating at 70° for 20 min were the same. This bound enzyme activity without soluble starch in the reaction mixture was about 35% lower than that incubated in the presence of soluble substrate.

Paper chromatography (PC). Analyses of degradation products from starch granules bound with alpha-amylase were performed by PC with *n*-BuOH–pyridine–H_2O (6 : 4 : 3) at room temp. for 20 hr. The developed PC strip was dried at 100–105° for 80 min. Sugars were detected by the procedure of ref. [31].

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