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PROPERTIES OF α-GLUCAN PHOSPHORYLASE FROM PEA CHLOROPLASTS

N. J. KRUGER* and T. AP REES

Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

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Key Word Index—*Pisum sativum*; Leguminosae; pea; chloroplasts; α-glucan phosphorylase; properties; ADPglucose in leaves.

Abstract—A partially purified preparation of α-glucan phosphorylase was obtained from chloroplasts of *Pisum sativum* by ion-exchange chromatography and gel filtration. The preparation, in which no other enzyme that metabolized starch or glucose 1-phosphate could be detected, was characterized. The optimum for phosphorolysis was pH 7.2; at pH 8.0 the activity was reduced by 50%. The preparation showed normal hyperbolic kinetics with the substrates, and catalysed the formation of [^{14}C]glucose 1-phosphate from ^{14}C-labelled starch grains from pea chloroplasts. None of the following, generally at 5 and 10 mM, significantly altered the rate of phosphorolysis: glucose, fructose, sucrose, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, ATP, ADP, AMP, 6-phosphogluconate, 2-phosphoglycollate, Mg^{2+}, dithiothreitol. However, phosphorolysis was inhibited by ADPglucose. Measurements of ADPglucose in leaves and in isolated chloroplasts showed that none could be detected in the dark and suggested that the concentration in the light was high enough to cause a modest inhibition of the phosphorylase. The control of the breakdown of chloroplast starch is discussed.

INTRODUCTION

The α-glucan phosphorylase (EC 2.4.1.1) in the chloroplasts of young pea leaves plays a central role in starch breakdown [1, 2]. There is evidence that the rate of starch breakdown in these leaves is much greater in the dark than in the light [3]. The following suggests that regulation of the phosphorylase contributes to the control of starch breakdown. First, the maximum catalytic activity of phosphorylase in the chloroplast, 0.022 μmol/min per mg chlorophyll, is not greatly in excess of the rate of net breakdown of starch *in vivo* [1]. Second, a ratio of glucose 1-phosphate:inorganic phosphate of 7.9×10^{-3} is obtained for chloroplasts in the dark if we use current estimates of stromal volume and metabolite content [4] and assume the latter to be confined to the stroma. This value is so much smaller than the apparent equilibrium constant (0.3–0.7) that it is likely that the phosphorylase reaction is significantly displaced from equilibrium in the chloroplast.

α-Glucan phosphorylase from spinach chloroplasts has been purified extensively [5, 6] and to homogeneity [7] and some of its properties have been described. The aim of the present work was to determine the properties of the phosphorylase from chloroplasts of pea leaves of the same age as those used to study starch breakdown [1, 3].

*Present address: Thimann Laboratories, University of California, Santa Cruz, CA 95064, U.S.A.

RESULTS AND DISCUSSION

Partial purification

In order to minimize damage to any labile regulatory properties, we purified the enzyme no further than was needed to give an unambiguous assay. The overall purification (Table 1) was a little greater than that reported for the spinach enzyme [5]. The first step in our purification was the isolation of chloroplasts. This separated the enzyme from much of the cell protein and protected it

Table 1. Partial purification of α-glucan phosphorylase from leaves of 14 to 18-day-old pea plants

Purification step	Volume (ml)	Total protein (mg)	Specific activity (pkat/mg protein)	Yield (%)	Purification (fold)
Leaf homogenate	1200	660	57	—	—
Chloroplast stroma	50	66.5	78	100	1.4
DEAE-cellulose	37	2.8	700	39	12.2
Ultrafiltration	3.5	2.7	741	39	13.0
BioGel P-150	10	1.5	1183	35	20.8

until it was free of the substances most likely to damage it: the contents of the vacuoles and compounds formed as a result of cell disruption. The carbon dioxide-dependent evolution of oxygen by the chloroplasts in the light was 99 μmol/hr per mg chlorophyll; 63% were intact and starch breakdown in the dark occurred at rates comparable to those in the leaf. We suggest that the initial homogenization did not seriously damage the chloroplast phosphorylase. The preparation contained, respectively, 0.7, 3 and 8% of the phosphoenolpyruvate carboxylase, fumarase and catalase found in the initial homogenates. This negligible contamination, and the evidence that chloroplasts from leaves of this age contain phosphorylase [1], demonstrate that our preparations of phosphorylase were from chloroplasts.

Using assays optimized for the crude homogenate of leaves we could detect none of the following in our preparations of phosphorylase: phosphatase activity with glucose 1-phosphate, α-amylase, β-amylase, phosphoglucomutase, ADPglucose and UDPglucose pyrophosphorylases. When the preparation was incubated with [^{14}C]amylopectin, as in the assay of phosphorylase, release of ^{14}C was completely dependent upon inorganic phosphate and [^{14}C]glucose 1-phosphate was the only product detectable. Similar incubations with [^{14}C]glucose 1-phosphate did not release detectable ^{14}C unless α-1,4-glucan was present. Thus, our preparations were free of contaminants capable of metabolizing either the product or the substrate of phosphorylase.

We avoided the use of coupled assays in our study of the properties of the enzyme. For phosphorolysis we stopped the reaction by boiling and measured glucose 1-phosphate. This did not cause significant losses of glucose 1-phosphate. Amounts of the latter equivalent to those formed in the assay were subjected to the killing procedure over the pH range 5.6–8.4, but no losses were detected. We showed that boiling completely inactivated the phosphorylase, and that activity was linearly related to time and the amount of protein.

Properties

We incubated the partially purified phosphorylase in 5 mM disodium hydrogen orthophosphate, pH 7.2, with six concentrations (0.01–20 g/l.) of a range of glucans. K_m^{app} for amylopectin, amylose, soluble starch and dextrins I–III were 0.104, 0.169, 0.079, 0.141, 0.103 and 0.118, respectively. The corresponding values for V^{app} were: 1.01, 0.82, 1.12, 0.71, 1.07 and 1.20 nkat/mg protein, respectively. The above were calculated from Lineweaver–Burk plots; similar values were obtained from direct linear plots [8]. Under the above conditions little or no activity was found with glycogen or pullulan. We chose amylopectin as the substrate for further studies of the phosphorylase.

The pH optimum in the synthetic direction was below 5.6; that for phosphorolysis, pH 7.2 (Fig. 1), corresponds to the stromal pH in the dark [9]. Phosphorolysis at pH 8.0, the stromal pH in the light, was about half that at pH 7.2. The optimum for phosphorolysis is sharper than that reported for the enzyme from spinach chloroplasts by Preiss *et al.* [5] but less marked and a good deal higher than that reported for the spinach enzyme by Shimomura *et al.* [6].

We investigated the effects of substrate concentration on phosphorolysis and synthesis of glucan at both pH 7.2 and 8.0. For phosphorolysis, phosphate was 0.5, 1, 2, 5 and

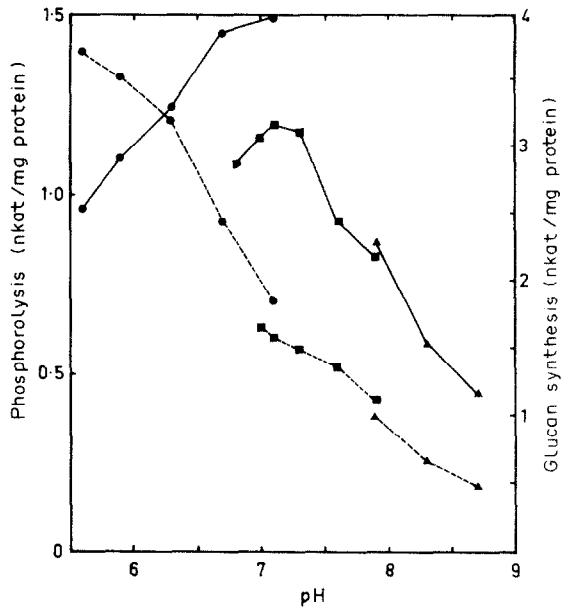

Fig. 1. Effect of pH on activity of partially purified phosphorylase. In the synthetic direction the reaction mixture contained, in 100 μl, 10 mM [U-^{14}C]glucose 1-phosphate (0.1 Ci/mol) and 0.9 mg amylopectin in 100 mM buffer; for phosphorolysis, in 400 μl, 15 mM Na_2HPO_4 and 0.2 mg amylopectin in 100 mM buffer. Buffers were imidazole–HCl (●), Tes–NaOH (■), and Tris–HCl (▲) in both phosphorolytic (————) and synthetic (– – –) directions. Variation in the concentration of buffers from 10 to 100 mM did not affect activity.

15 mM; and for synthesis, glucose 1-phosphate was 1, 2, 4, 10 and 20 mM. In both directions amylopectin was 0.05, 0.1, 0.5, 1.0 and 5.0 g/l. K_m^{app} and V^{app} were calculated by linear regression analysis of Lineweaver–Burk plots. For each analysis the correlation coefficient was greater than 0.985. This, and the fact that the gradients of Hill plots for each set of data were within the range 0.9–1.1, provide adequate evidence that the phosphorylase shows hyperbolic kinetics.

For each substrate we determined the Michaelis constant at saturating levels of the second substrate (K_m), the dissociation constant in the absence of the second substrate (K_i), and the maximum velocity at saturating concentrations of both substrates (V). The values (Table 2) were obtained from secondary plots of b/V^{app} and bK_m^{app}/V^{app} vs. b as described by Cornish-Bowden [10], where b is the concentration of the second substrate. This analysis assumes that the enzyme operates via a rapid equilibrium random bi bi mechanism, as has been proposed for glucan phosphorylases from other sources [11]. The affinity of the enzyme for its substrates is not markedly affected by pH. The absolute values of the constants are comparable to those reported for the enzyme from spinach chloroplasts [11].

To assess the role of the phosphorylase we need to know if it can attack chloroplast starch grains. We investigated this problem by determining whether the phosphorylase released ^{14}C from labelled starch grains. The latter were from pea chloroplasts since starch grains from different sources may vary in their susceptibility to digestion [12]. We incubated chloroplasts from 7-day-old

Table 2. Kinetic parameters of partially purified phosphorylase

Direction	Constant	Value pH 7.2	Value pH 8.0	Units
Phosphorolysis	K_mPi	0.45	0.47	mmol/l.
	K_iPi	4.36	3.98	mmol/l.
	K_m amylopectin	0.042	0.049	g/l.
	K_i amylopectin	0.433	0.429	g/l.
	V	1.32	0.92	nkat/mg protein
Synthesis	K_m Glc-1-P	0.96	1.07	mmol/l.
	K_i Glc-1-P	5.09	5.13	mmol/l
	K_m amylopectin	0.078	0.092	g/l.
	K_i amylopectin	0.411	0.433	g/l.
	V	1.97	1.16	nkat/mg protein

Values are means of three estimates: s.e. was less than 5% of mean.

plants in sodium hydrogen [^{14}C]carbonate in the light for 15 min. Evidence that this labelled starch is provided by the fact that 95% of the ^{14}C in the water-insoluble material was recovered as [^{14}C]glucose after digestion with amyloglucosidase and α-amylase. Next, intact chloroplasts were isolated from the labelled preparations by sucrose density centrifugation and were immediately lysed in water to give starch grains that were isolated by centrifugation. The grains contained at least 90% of the ^{14}C present in the water-insoluble fraction at the end of the incubation in sodium hydrogen [^{14}C]carbonate. We argue that these labelled grains originated only from intact chloroplasts and, thus, had not been damaged mechanically. First, little starch would have been made from sodium hydrogen [^{14}C]carbonate by broken chloroplasts. Second, the centrifugation effectively separates intact chloroplasts from broken chloroplasts and starch grains [1]. It is also unlikely that the labelled grains had been attacked by α- or β-amylase. Less than 2% of the activity of these enzymes in the initial homogenate is recovered in the crude preparations of chloroplasts, and this contamination is removed on the density gradient [1].

The ability of pea chloroplast phosphorylase to degrade the labelled starch grains was compared to those of pure α-amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41) (Table 3). To check that the grains did not contain inhibitors of these enzymes we compared [^{14}C]amylopectin, labelled grains, and a mixture of the two as substrates. Comparison of the activities of the mixture with those predicted from the activities of the separate components of the mixture (Table 3) shows that it is most unlikely that the grains contained inhibitors of the enzymes. There was a small loss of ^{14}C from grains incubated without added enzyme. This was not due to enzymic contamination of the grains as it was not prevented by previously incubating the grains at 100° for 5 min and was also found with [^{14}C]amylopectin.

Incubation of the labelled starch grains with the phosphorylase preparation converted 80% of the labelled starch into soluble form. Chromatography showed that over 90% of the ^{14}C released co-purified with glucose 1-phosphate. Phosphorylase released more label than either amylase or pullulanase; neither of the latter acted synergistically with the phosphorylase. Omission of in-

Table 3. Release of ^{14}C from labelled starch grains and amylopectin incubated with phosphorylase, α-amylase and pullulanase

Addition	^{14}C released (dpm/sample) Grains	Amylopectin	Grains + amylopectin	Release by mixture as % that predicted
Pi	152 ± 38	200 ± 9	335 ± 5	95 ± 2
Phosphorylase + Pi	2010 ± 21	2069 ± 55	3794 ± 15	93 ± 1
Phosphorylase	1884 ± 5	199 ± 13	2863 ± 27	137 ± 1
α-Amylase + Pi	1337 ± 95	1186 ± 27	2518 ± 179	100 ± 8
Phosphorylase + Pi + α-amylase	1916 ± 39	2005 ± 41	3560 ± 103	91 ± 3
Pullulanase + Pi	1019 ± 141	1042 ± 29	2080 ± 129	101 ± 6
Pullulanase + Pi + phosphorylase	2050 ± 12	2051 ± 62	3966 ± 79	97 ± 2
Total ^{14}C present at start of incubation	2554 ± 3	3682 ± 14	6218 ± 27	—

Samples were incubated at 25° for 18 hr in 400 μl 100 mM Tes (pH 7.2) that contained 1 μg labelled starch or 4 μg labelled amylopectin, or 1 μg labelled starch + 4 μg labelled amylopectin. Additions were 10 mM disodium hydrogen orthophosphate, phosphorylase (0.027 unit), α-amylase (0.020 unit), pullulanase (0.03 unit). Values are means ± s.e. from three samples.

organic phosphate from the incubation mixture did not greatly decrease the release of ^{14}C from starch grains by the phosphorylase but abolished release from [^{14}C] amylopectin. Analysis of the grains and the amylopectin showed that their inorganic phosphate content would give concentrations in the reaction mixtures of 45 and 3 μM, respectively. This suggests that the release of ^{14}C from the grains in the absence of added inorganic phosphate was due to phosphorolysis at the expense of the inorganic phosphate in the preparation of grains. This view is supported by the fact that all the ^{14}C released in the absence of added inorganic phosphate co-chromatographed with glucose 1-phosphate. Further, release from the mixture of starch grains and amylopectin in the absence of added inorganic phosphate exceeded that expected (Table 3). This would occur if there had been enough inorganic phosphate in the grains to allow phosphorolysis of the amylopectin as well as the grain. Lysis of the purified chloroplasts in 10 mM mercuric chloride, instead of water, did not diminish the ability of the phosphorylase to release label from the starch grains. This is further evidence that the release of ^{14}C was not due to contaminating amylases. We suggest that phosphorylase from pea chloroplasts can release glucose 1-phosphate from chloroplast starch grains, and that this does not depend upon mechanical damage to the grains or enzymic attack by conventional α- or β-amylases.

The effects of light on the distribution of Mg^{2+} in the chloroplast led us to determine the response of phosphorylase to 2, 5 and 10 mM magnesium acetate at pH 7.2 and 8.0 and 0.1 g/l. amylopectin and at inorganic phosphate concentrations of 0.5, 1, 2, 5 and 15 mM. No response to Mg^{2+} was found. We investigated whether the phosphorylase was modulated by a light-generated reductant. We incubated the enzyme at 25° for 15 min in 10 and 20 mM dithiothreitol and then assayed the activity. Dithiothreitol was included in the assay at the concentration used in the pre-treatment and the assay mixture was varied as in the experiments with Mg^{2+}. We found no response to dithiothreitol. In further experiments, crude preparations of chloroplasts were incubated for 15 min at 25° in the dark; samples were then lysed and incubated for a further 15 min in the dark in 20 mM dithiothreitol or water. The treatment with dithiothreitol caused the expected [13] substantial (75%) inhibition of glucose-6-phosphate dehydrogenase but did not alter the activity of the phosphorylase. The above, and the evidence for phosphorolysis of chloroplast starch in the light [14], make it unlikely that chloroplast phosphorylase is modulated directly by light.

We investigated the effects of a wide range of compounds on the phosphorolytic activity of the preparation. Each compound was tested at five concentrations (0.5, 1, 2, 5 and 15 mM) of inorganic phosphate, and 0.1 g/l. amylopectin, and at both pH 7.2 and 8.0. The compounds were: 10 mM sucrose, 5 mM fructose 6-phosphate; the following at 5 and 10 mM; fructose 1,6-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, 6-phosphogluconate, ADP and AMP; ATP at 0.2, 2.5 and 10 mM; 2 and 5 mM 2-phosphoglycollate. The correlation coefficients of linear analysis of Lineweaver–Burk plots for each set of data were > 0.985. Phosphorolysis under each of the above conditions was always within 5% of that of untreated controls. The failure of these compounds to affect phosphorolysis was not due to their metabolism by the phosphorylase preparation. We incubated the latter with each of the compounds at 1 mM at pH 7.2 for 30 min at 25° and measured whether this caused loss of the compounds. No significant losses were found. To investigate whether the phosphorylase is modified by a chloroplast protein kinase we incubated a crude preparation of chloroplasts in the light for 15 min, lysed the chloroplasts and immediately assayed the phosphorylase in the lysate in the absence and presence of 5 and 10 mM ATP. No effect was found.

ADPglucose significantly inhibited, 60% at 4 mM, pea chloroplast phosphorylase (Fig. 2). We determined the effects of ADPglucose on the affinity of the enzyme for inorganic phosphate and amylopectin at pH 7.2 and 8.0. The data obtained at pH 8.0 (Figs. 3 and 4) do not differ significantly from those obtained at pH 7.2. The uncompetitive-inhibition constants at pH 8.0 [10], calculated from these data are 2.35 mM for inorganic phosphate (amylopectin at 0.1 g/l.) and 2.33 mM for amylopectin (inorganic phosphate at 1 mM). The competitive-inhibition constants [10], calculated for the same conditions are 0.46 and 0.59 mM, respectively. The data suggest that ADPglucose is a mixed inhibitor with respect to both substrates. By mixed inhibition we mean inhibition that is not due solely to increased K_m, or to decreased V, or the K_m and V being decreased in constant ratio, but to some combination of the above factors.

Effects of light on amounts of ADPglucose in leaves and chloroplasts

We did these experiments to see if any changes in ADPglucose content could contribute to the regulation of

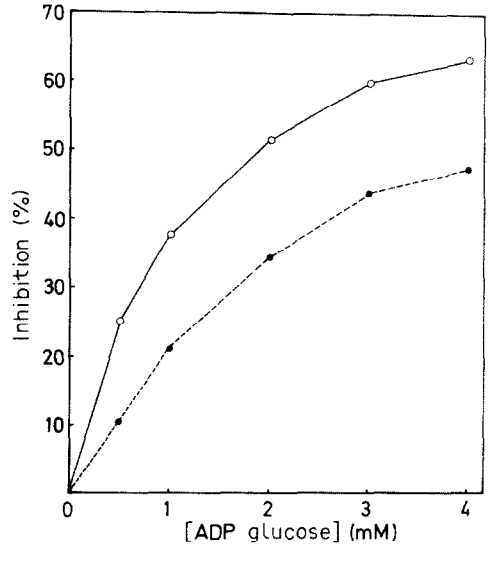

Fig. 2. Effects of ADPglucose on the activity of partially purified phosphorylase. The reaction mixture contained, in 400 μl, 100 mM Tes (pH 8.0), 5 mM Na_2HPO_4, 0–4 mM ADPglucose and either 0.04 mg (O——O) or 0.4 mg (●- - - -●) amylopectin. At each concentration of ADPglucose the difference between the rates in its presence and absence is expressed as a percentage of the rate in its absence to give percentage inhibition. Each point is the mean of estimates from three samples: s.e. was less than 5% of mean.

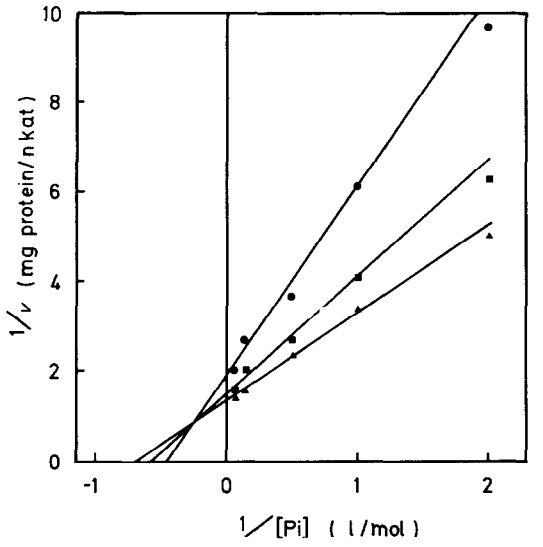

Fig. 3. Effect of phosphate concentration on activity of partially purified phosphorylase in the presence of ADPglucose. The reaction mixture contained in 400 μl: 100 mM Tes (pH 8.0), 0.04 mg amylopectin, 0.15–15 mM Na_2HPO_4, and no (▲), 0.5 mM (■) and 1.0 mM (●) ADPglucose. Data are presented as Lineweaver–Burk plots. Each point is the mean of estimates from three samples: s.e. was less than 5% of mean.

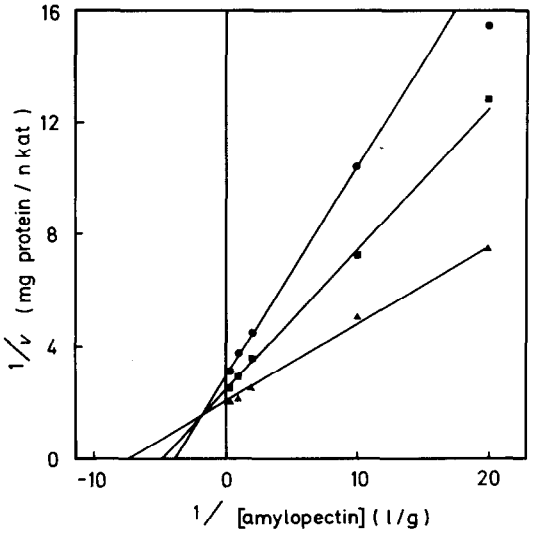

Fig. 4. Effect of amylopectin concentration on activity of partially purified phosphorylase in the presence of ADPglucose. The reaction mixture contained in 400 μl: 100 mM Tes (pH 8.0), 1 mM Na_2HPO_4, 0.02–2.0 mg amylopectin, and no (▲), 0.5 mM (■) and 1.0 mM (●) ADPglucose. Data are presented as in Fig. 3.

chloroplast phosphorylase *in vivo*. Although there is evidence that the amount of ADPglucose in *Chlorella* rises rapidly on illumination [15], no data appear to be available for leaves. We used freeze-clamping to ensure immediate cessation of metabolism, isolated ADPglucose from the resulting extracts by HPLC and measured it by its absorbance. The following experiments authenticate this procedure. Pure ADPglucose was eluted from the column as a single reproducible peak with an R_t of 28.1 ± 1 min (mean ± s.e. of 15 measurements). In the range in which we worked, 0.06–16 nmol/sample, the maximum absorbance of this peak was linearly related to the amount of ADPglucose added to the column. Under the same conditions the R_ts (min) of related compounds were: AMP, 12.3; ADP 36.3; ATP, 47.3; CDPglucose, 26.2; UDPglucose, 27.6; UDPmannose, 27.7; UDPxylose, 27.9; ADPmannose, 28.0; ADPribose, 28.6; GDPglucose, 30; UDPglucuronic acid, 32.8. Co-chromatography of the above compounds plus ADPglucose confirmed that the latter was well separated from the adenine nucleotides and UDPglucuronic acid but not from some of the sugar nucleotides.

When extracts of pea leaves were chromatographed UDPglucose obscured ADPglucose. Treatment of the extract with UDPglucose dehydrogenase (EC 1.1.1.22) converted the UDPglucose to UDP glucuronic acid, which separated from ADPglucose to reveal a peak of the latter. The following is evidence that this peak of ADPglucose in the extracts was not significantly contaminated. The UV absorption spectrum of the peak differed from that of compounds with similar R_ts and was similar to that of authentic ADPglucose (λ_{max} 259, A_{280}/A_{254} 0.197). The peak of ADPglucose from leaves was hydrolysed and accounted for as comparable amounts of glucose and adenine (Table 4).

We investigated whether ADPglucose was lost during killing, extraction and analysis of the leaves. For each test we took duplicate samples of leaves. One was freeze-clamped, killed in perchloric acid, extracted and analysed: the other was treated similarly except that 10 nmol ADPglucose was added to the perchloric acid with the freeze-clamped leaves. Comparison of the two samples allowed calculation of the recovery of the added ADPglucose. The recoveries were satisfactory (Tables 5 and 6). In similar experiments we established that 2 nmol ADPglucose/g fr. wt was the lowest amount that could be recovered satisfactorily from samples of leaves. We investigated whether cutting the leaves off the plant for the preparation of the samples affected their content of ADPglucose. We found no differences between the ADPglucose content of illuminated leaves freeze-clamped on the plant and those freeze-clamped up to 1 min after

Table 4. Adenine and glucose content of ADPglucose fraction isolated by HPLC from pea leaves

Fraction eluted from column ADPglucose content (nmol)	Hydrolysate of eluted fraction	
	Adenine (nmol)	Glucose (nmol)
2.18	2.12	2.29
1.27	1.13	1.10
2.68	2.33	2.38

Date are analyses of ADPglucose fractions from extracts of leaves from three batches of separately grown plants. The eluted fraction was hydrolysed by incubation with 0.1 M hydrochloric acid at 100° for 10 min. After neutralization with 1 M sodium hydroxide adenosine was measured by A_{259}, and glucose as in ref. [16].

Table 5. ADPglucose content of pea leaves

Pre-treatment of leaves	ADPglucose (nmol/g fr. wt)	Recovery of added ADPglucose (%)
9 hr light	27.5 ± 2.2 (5)	81 ± 2 (3)
9 hr light then 0.5 hr dark	< 2 (5)	93 ± 5 (3)
18 hr light	28.6 ± 3.1 (5)	90 ± 4 (3)
18 hr light then 0.5 hr dark	< 2 (5)	85 ± 3 (3)

Values are means ± s.e. of estimates from the number of samples, each from a separately grown batch of peas, shown in parentheses.

detachment from the plant. We conclude that the method for measuring ADPglucose is reliable.

There were 28 nmol ADPglucose per g fr. wt in pea leaves that had been illuminated for 9 and 18 hr (Table 5). We could detect no ADPglucose in darkened leaves and argue that their content was less than 2 nmol/g fr. wt. We also measured ADPglucose in isolated chloroplasts in which we had varied the rate of starch breakdown (Table 6). We incubated the chloroplasts in the light in sodium hydrogen [^{14}C]carbonate for 15 min to label the starch and then for 15 min in sodium hydrogen [^{12}C]carbonate in the light to reduce the amount of ^{14}C in soluble compounds in the stroma. Next, samples of the labelled chloroplasts were incubated for 30 min in high phosphate medium in the light and dark, and in low phosphate medium in the light. Starch breakdown was measured by determining the loss of ^{14}C from starch during the 30-min incubation, and ADPglucose was measured at the end of the 30 min (Table 6). There was considerable breakdown of starch in the high phosphate medium in both the dark and light but very little breakdown in the low phosphate medium in the light. No ADPglucose was detected where there was appreciable breakdown of starch but it was found where starch breakdown was restricted.

Conclusions

Our preparations of chloroplast phosphorylase were pure enough to permit accurate and direct assay of the enzyme, and were obtained without exposing the enzyme to harsh conditions. Our evidence that the enzyme can attack chloroplast starch grains without the intervention of conventional α- or β-amylase is consistent with the distribution of these enzymes in pea leaves [1], and provides further support for the importance of phosphorolysis in the breakdown of starch in pea chloroplasts. Our studies of the effects of pH tend to confirm the view that choice of buffer influences the pH optimum. However, we did find optimal phosphorolysis at the pH of the stroma in the dark, and that raising the pH to that of the stroma in the light causes a substantial reduction in phosphorolysis. We found no significant evidence that pea chloroplast phosphorylase has any sophisticated regulatory properties comparable to those of glycogen phosphorylase. The methods we used to prepare the enzyme, and the wide range of conditions under which we studied it, make it unlikely that regulatory properties were either lost during the isolation or not included in the characterization. The hyperbolic kinetics and the failure to respond to most metabolites are also characteristic of the enzyme from spinach chloroplasts [5–7].

Pea chloroplast phosphorylase is inhibited by ADPglucose. Shimomura *et al.* [6] reported a less marked effect of ADPglucose on spinach chloroplast phosphorylase whilst none was reported by Preiss *et al.* [5]. These studies of the effects of ADPglucose on the spinach chloroplast phosphorylase are not extensive enough to show whether it differs in this respect from the enzyme from pea chloroplasts. Our measurements of ADPglucose in leaves and chloroplasts demonstrate striking effects of light on the content of ADPglucose, and show that the latter is inversely related to starch breakdown. This poses the question of whether ADPglucose contributes to the control of starch breakdown by modulating phosphorolysis. Hall [17] has estimated that the volume of stroma of isolated chloroplast is 25 μl/mg chlorophyll. If we assume that all of the ADPglucose in the leaf is in the stroma of the chloroplast, then the data in Table 5 suggest that the concentration of ADPglucose in the stroma in the light would be close to 0.4 mM. We showed that 85% of the chloroplasts, referred to in Table 6, were intact. Thus, the concentration of ADPglucose in the stroma of the chloroplasts in low phosphate in the light may be calculated to have been close to 0.4 mM. From Fig. 2 it can be seen that 0.4 mM ADPglucose would only reduce the activity of the phosphorylase by about 25%. This suggests that, whilst changes in ADPglucose can contribute to the regulation of chloroplast starch breakdown in peas, such changes do not play a dominant role in the regulation.

The picture of starch breakdown in pea chloroplasts suggested by the present and previous [3] studies closely complements that presented for chloroplasts isolated from spinach by Stitt and Heldt [14]. Breakdown does

Table 6. ADPglucose content of chloroplasts having different rates of starch breakdown

Incubation conditions	Loss of ^{14}C from starch as % of that present at start of incubation	ADPglucose (nmol/mg chlorophyll)	Recovery of added ADPglucose (%)
Low phosphate			
Dark	−53 ± 3 (4)	< 0.8 (6)	88 ± 2 (4)
Light	−52 ± 4 (4)	< 0.8 (6)	83 ± 1 (4)
High phosphate			
Light	−2 ± 1 (4)	7.9 ± 0.6 (6)	88 ± 2 (4)

Values are means ± s.e. from a number (given in parentheses) of separate preparations of chloroplasts.

not appear to be controlled directly by light or, to any great extent, by any allosteric or regulatory properties of α-glucan phosphorylase. It is possible that the concentration of inorganic phosphate in the stroma is of prime importance and that this mechanism is supplemented by changes in pH and ADPglucose.

EXPERIMENTAL

Material. Substrates, co-factors and enzymes were from Boehringer, except fructose 6-phosphate and dextrins I–III from Sigma and amylopectin from Koch-Light. DEAE-cellulose and BioGel P-150 were from BioRad Laboratories, Bromley, U.K. Peas (*Pisum sativum* L. cv Kelvedon Wonder) were grown in an 18-hr photoperiod but otherwise as in ref. [18].

Chloroplasts. These were made from the shoots of 6 to 8-day-old plants as in ref. [19] and from the leaves of 14 to 18-day-old plants as in ref. [1]. The preparations obtained are referred to as crude preparations. Unless specified otherwise the chloroplasts were resuspended in 50 mM Hepes (pH 7.6), 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.1% (w/v) bovine serum albumin. Chloroplasts were purified, and the reliability of the purification demonstrated by assaying each fraction for the appropriate marker enzymes, exactly as in ref. [1], which also gives the methods used to measure the intactness and oxygen evolution of the chloroplasts.

Partial purification of α-glucan phosphorylase. We amalgamated 8–10 preparations of chloroplasts from 14 to 18-day-old plants, lysed them by resuspension in 20 mM citrate buffer and centrifuged the lysate at 15000 *g* for 15 min. We put the supernatant on a column (17 × 2.4 cm) of DEAE-cellulose, equilibrated with 20 mM citrate buffer, pH 6.5, and then eluted with, successively, 50 ml 20 mM citrate buffer (pH 6.5), a 100-ml linear gradient from 20 to 60 mM citrate buffer (pH 6.5), and a 100-ml linear gradient from 0 to 0.5 M NaCl in 60 mM citrate buffer (pH 6.5). Fractions of 8 ml were collected and those from the major peak of activity that eluted in the NaCl gradient were concd to 3 ml with an Amicon ultrafiltration system (membrane, Diaflo PM 10), and then put on a column (45 × 2.5 cm) of BioGel P-150 equilibrated with 20 mM citrate buffer (pH 6.5). The same buffer was used for elution; the fractions (3 ml) that contained the phosphorylase were collected to give the partially purified preparation.

Isolation of starch grains. Crude preparations of chloroplasts from 7-day-old plants were incubated, at 0.1 mg chlorophyll/ml, in 10 ml 5 mM $Na_4P_2O_7$, 0.1 mM Na_2HPO_4, 0.8 mM ATP, 10 mM $NaH^{14}CO_3$ (0.5 μCi/μmol) for 15 min at 20° in the light (1.06 J/min per cm^2). The chloroplasts were then sedimented (4000 *g* for 30 sec), washed twice in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.1% (w/v) albumin, and then incubated as above except that the bicarbonate was not labelled. They were sedimented, washed again and resuspended in 30% (w/w) sucrose, 100 mM Tricine (pH 7.6), 10 mM KCl, 1 mM $MgCl_2$, 5 mM 3-phosphoglycerate, 0.1% albumin, 1% (w/v) Ficoll, 1% (w/v) Dextran T40. Intact chloroplasts were isolated from this suspension by density gradient centrifugation as in ref. [1], and then lysed by diluting × 10 with H_2O. The lysate was centrifuged at 10000 *g* for 5 min and the sediment washed × 3 by resuspension in H_2O and centrifugation (20000 *g*, 2 min). The final sediment was used as starch grains.

Starch breakdown. Labelled chloroplasts were prepared and treated as above except that they were resuspended, not in 30% sucrose etc., but in 50 mM Hepes (pH 7.6), 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.1% albumin that contained either 1 mM ATP and 7.5 mM Na_2HPO_4 (high phosphate) or 10 mM $NaHCO_3$, 5 mM $Na_4P_2O_7$, 0.8 mM ATP and 0.1 mM Na_2HPO_4 (low phosphate). A sample (2.0 ml) of each suspension was made 1.41 M with $HClO_4$ at once. Replicate samples were incubated at 25° for 30 min in the light (1.06 J/min per cm^2) or dark and then made 1.41 M with $HClO_4$. All samples were kept in the $HClO_4$ for 4 hr at 2° and then centrifuged (4000 *g*, 10 min). The sediment was washed twice in H_2O and its content of ^{14}C used as a measure of the labelling of starch. Treatment of this sediment with hydrolases as in ref. [20] released over 97% of the ^{14}C as [^{14}C]glucose.

Enzyme assays. α-Glucan phosphorylase in crude extracts and during purification was measured by the coupled assay in ref. [1]. For the partially purified enzyme the basic assays were: phosphorolysis, enzyme (20–25 μg protein), 100 mM Tes (pH 7.2 or 8.0), 15 mM Na–KPi and 2 mg amylopectin were incubated in 400 μl for 30 min at 30°, heated to 100° for 40 sec and then assayed spectrophotometrically for glucose 1-phosphate as in ref. [16]; synthesis, as in ref. [21] in 100 μl that contained 10–12 μg protein, 100 mM Tes (pH 7.2 or 8.0), 10 mM [U-^{14}C]glucose 1-phosphate (0.1 μCi/μmol), 0.9 mg amylopectin. α-Amylase (EC 3.2.1.1), the preparation was made 11 mM with $Ca(OAc)_2$ (pH 6.0) and assayed as in ref. [22]. ADPglucose pyrophosphorylase (EC 2.7.7.27) was assayed as in ref. [23] in 200 μl that contained: 100 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 3.5 mM 3-phosphoglycerate, 0.68 mM [U-^{14}C]glucose 1-phosphate (0.5 μCi/μmol), 50 μg albumin, 0.33 μg crystalline yeast inorganic pyrophosphatase (EC 3.6.1.1), 1.2 mM ATP. UDPglucose pyrophosphorylase (EC 2.7.7.9) was assayed similarly except that UTP was used instead of ATP and 3-phosphoglycerate was omitted. Phosphatase (EC 3.1.3.1) was assayed as in ref. [24] in 50 mM sodium citrate buffer (pH 6.0), 2.5 mM *p*-nitrophenyl phosphate in 200 μl. The assays for β-amylase (EC 3.2.1.2) and phosphoglucomutase (EC 2.7.5.1) are in ref. [1].

Assay of metabolites. For ADPglucose in leaves, 1 g fr. wt was freeze-clamped within 15 sec of harvesting and killed and extracted as in ref. [25] except that the period in $HClO_4$ prior to neutralization was reduced to 30 min. Samples (1 ml) of the neutralized extract, equivalent to 200 mg fr. wt, were incubated with 3 mM NAD and 0.03 unit UDPglucose dehydrogenase (EC 1.1.1.22) for 1 hr at 30°. This mixture was then passed through a Millipore filter (0.45 μm) and 10–50 μl injected into a Spectra-Physics HPLC system (SP 8700 solvent delivery system, SP 8300 UV detector, Rheodyne model 7125 sample injector) fitted with a Partisil-10-SAX column (0.46 × 25 cm). The column was eluted with, successively, 12 ml 10 mM $NH_4H_2PO_4$ (pH 3.0), 12 ml of a linear gradient from 10 mM (pH 3.0) to 450 mM (pH 4.3) $NH_4H_2PO_4$, 12 ml 450 mM $NH_4H_2PO_4$ (pH 4.3). Flow rate was 0.8 ml/min and operating pres. was 4.14–4.49 MPa. Eluted compounds were detected by A_{254}. ADPglucose was measured by comparing A_{254} of the eluted peak with a calibration curve made from measurements of 0.06–15.8 nmol authentic ADPglucose eluted from the column. ADPglucose in chloroplasts was measured as above except that the chloroplasts were treated with $HClO_4$ without freeze-clamping, and the treatment with UDPglucose dehydrogenase was omitted. To determine whether possible effectors of the phosphorylase survived incubation with the partially purified enzyme, the phosphorylase reaction mixtures were made 1.41 M with respect to $HClO_4$, left at −2° for 30 min, neutralized as above, centrifuged and the supernatant assayed spectrophotometrically as in ref. [16] except for 3-phosphoglycerate which was assayed as in ref. [26].

Miscellaneous. [^{14}C]Amylopectin was made by incubating 100 mg amylopectin in 25 mM imidazole buffer (pH 7.1), 1.3 mM [^{14}C]glucose 1-phosphate (0.12 μCi/μmol), 3 units rabbit muscle α-glucan phosphorylase *a* in 3 ml for 6 hr at 37°, and then heating to 100° for 5 min, dialysing against four changes, each of 8 hr, of

150 ml 150 mM imidazole buffer (pH 7.1). The non-dialysable material was taken as [^{14}C]amylopectin. The following were measured as in the accompanying references: chlorophyll [27], phosphate [4], protein [28], ^{14}C [2].

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