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Mode of glucan degradation by purified phosphorylase forms from spinach leaves

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Abstract. The glucan specifity of the purified chloroplast and non-chloroplast forms of α-1,4-glucan phosphorylase (EC 2.4.1.1) from spinach leaves (Steup and E. Latzko (1979), Planta **145**, 69–75) was investigated. Phosphorolysis by the two enzymes was studied using a series of linear maltodextrins (degree of polymerization $\leqq 11$), amylose, amylopectin, starch, and glycogen as substrates. For all unbranched glucans (amylose and maltodextrins G_5–G_{11}), the chloroplast phosphorylase had a 7–10-fold higher apparent affinity (determined by initial velocity measurements) than the non-chloroplast phosphorylase form. For both enzyme forms, the minimum chain length required for a significant rate of phosphorolysis was five glucose units. Likewise, phosphorolysis ceased when the maltodextrin was converted to maltotetraose. With the chloroplast phosphorylase, maltotetraose was a linear competitive inhibitor with respect to amylose or starch ($K_i = 0.1$ mmol l^{-1}); the inhibition by maltotetraose was less pronounced with the non-chloroplast enzyme. In contrast to unbranched glucans, the non-chloroplast phosphorylase exhibited a 40-, 50-, and 300-fold higher apparent affinity for amylopectin, starch, and glycogen, respectively, than the chloroplast enzyme. With respect to these kinetic properties the chloroplast phosphorylase resembled the type of "maltodextrin phosphorylase".

Key words: Amylopectin – Amylose – Glucan phosphorylase – Glycogen – Maltodextrin – *Spinacia* – Starch.

Introduction

During photosynthesis a considerable proportion of fixed carbon is usually stored inside the chloroplast as starch granules which may be degraded during the following dark period. At present, the degradation of this transitory starch and the regulation of this process are incompletely understood. However, there is some evidence that both amylase and phosphorylase are involved in the mobilization of chloroplast starch: (1) free sugars (glucose and maltose) and phosphorylated intermediates accumulated during starch degradation in preparations of isolated intact chloroplasts (Peavey et al. 1977; Stitt and ap Rees 1980); (2) orthophosphate stimulated the mobilization of chloroplast starch (Heldt et al. 1977; Steup et al. 1976), and (3) amylase and phosphorylase activity has been found in isolated chloroplasts (Pongratz and Beck 1978; Steup and Latzko 1979). These observations raise the question of whether amylolysis and phosphorolysis form two pathways of degradation, acting independently of each other, or, alternatively, whether amylase and phosphorylase activities closely cooperate within the same pathway of granule degradation. Obviously, the precise function of amylase and phosphorylase activity in starch degradation has to be clarified before an adequate understanding of the regulatory mechanisms which may control the mobilization of the stored carbohydrate can be achieved.

As a complication, phosphorylase activity (as well as that of other starch-degrading enzymes) often appears to be heterogenous; different phosphorylase forms have been observed inside and outside the chloroplast or even within this organelle (Steup and Latzko 1979). Spinach leaves contain one chloroplast and one non-chloroplast phosphorylase form, separable from each other by polyacrylamide gel electrophoresis or ion-exchange chromatography (Steup et al. 1980a). For a better understanding of the physiological function of the two enzyme forms, the glucan specifity of the two phosphorylases was studied. In the following, we report on kinetic properties of the two purified

Abbreviations: G1P = Glucose 1-phosphate; MES = 2(N-morpholino)ethane sulphonic acid; P_i = orthophosphate; Tris = Tris(hydroxymethyl)aminomethane

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phosphorylase forms as determined by initial velocity measurements. Unbranched glucans (amylose and a series of maltodextrins of increasing chain lengths) and branched glucans (amylopectin, starch, glycogen) were used as substrates for phosphorolysis. A preliminary report of some of these data has appeared previously (Steup et al. 1981).

Materials and Methods

Plant materials. For phosphorylase purification, spinach (*Spinacia oleracea* L.) was purchased from the local market. Intact chloroplasts were isolated from plants grown in water cultures (for details see Steup and Latzko 1979).

Phosphorylase purification. The two phosphorylase forms were purified as described elsewhere (Steup et al. 1980b; Steup 1981). The phosphorylase activity, however, was eluted from the Sepharose-glucan gels with a dextrin preparation containing less than 15 glucose units per molecule. This sample was prepared from a commercial dextrin (Serva, Heidelberg, FRG) by removing larger dextrins [precipitation with 75% (v/v) ethanol; the precipitate was discarded]. Dextrins were removed from the phosphorylase preparations by gel filtration (Sephadex G-100). Following this step, no phosphorolytic or glucan synthesizing activity in the enzyme preparations was detectable in the absence of an added glucan. The two phosphorylase preparations were free of cross-contamination as revealed by SDS gel electrophoresis and isoelectric focussing.

Phosphorylase assay. Phosphorolytic activity was determined as described elsewhere (Steup and Latzko 1979) except that Na_2MoO_4 was omitted and glucans were added as indicated. Activities were calculated from changes in absorbance of the reaction mixture at 340 or 365 nm. For the two enzymes the pH optimum of phosphorolytic activity was determined using three buffers (MES-NaOH pH 5.8–6.7; imidazol-HCl pH 6.4–7.8; Tris-HCl pH 7.3–8.4) and starch or amylose as the substrate. With these two substrates, both enzymes exhibited highest activity around pH 7.0. Therefore, for kinetic measurements all assays were performed using 25 mmol imidazol l^{-1}, adjusted to pH 7.0 with HCl, as the buffer.

Glucan synthesizing activity was determined by measuring the release of orthophosphate according to Bartlett (1959). All phosphorylase assays were performed at 30° C.

Glucans. Starch (Merck, Darmstadt, FRG), amylopectin (from potato, purchased from Sigma, München, FRG), and glycogen (from molluscs, obtained from Boehringer, Mannheim, FRG) were dialyzed against water prior to use. Amylose (EGA, Steinheim, FRG) consisted of polyglucans with molecular weights ranging from 1,500–8,000. For quantitation, aliquots of these glucans were hydrolyzed enzymatically (treatment with amyloglucosidase from *Aspergillus niger,* purchased from Boehringer, Mannheim, FRG) and the glucose liberated was determined by the hexokinase/glucose 6-phosphate dehydrogenase assay.

Maltodextrins (G_4-G_{11}) were separated by gel filtration (BIO-Gel P-4, 200–400 mesh; column 20 $cm^2 \cdot 150$ cm; flow rate 5 cm h^{-1}; 50° C) according to John et al. (1969). Commerical dextrin (Serva, Heidelberg, FRG), after a pretreatment with pullulanase (from *Aerobacter aerogenes,* Boehringer, Mannheim, FRG), served as the starting material. In the eluate, sugars were monitored by refractive index detection. Each maltodextrin was pooled separately and rechromatographed. By the same gel filtration procedure commercial samples of glucose, maltose, and maltotriose were separated from contaminants. The purified maltodextrins were identified by thin-layer chromatography with authentic oligosaccharides.

Thin-layer chromatography. A modification of the procedure described by Kanaya et al. (1978) was used (Silica plates "Polygram" Sil G/UV 254 from Machery and Nagel, Düren, FRG). Chromatograms were developed twice in a mixture of butanol, isopropanol, and water (7:8:5 by vol.). After chromatography, compounds were detected by sulfuricacid treatment (9 mol H_2SO_4 l^{-1}, 10–30 min at 120° C) or by treatment with N-(1-naphthyl)ethylenediamine (Bounias 1980).

Results

Phosphorolysis of amylose and maltodextrins. With the purified chloroplast and non-chloroplast phosphorylase, the initial velocity of glucose 1-phosphate formation was determined using varying amylose concentrations at different fixed orthophosphate levels. Amylose was quantified as glucose equivalents after exhaustive hydrolysis (see Materials and Methods). In the coupled enzyme assay, increase in absorbance at 340 or 365 nm was constant for at least 5 min with both phosphorylase forms. When the initial reaction velocity (v) was plotted against the amylose concentration (S_1), a series of hyperbolic saturation curves was obtained for both enzymes (data not shown). Double reciprocal plots of the data were linear and showed converging line patterns with a crossover point above the horizontal axis (Fig. 1). For the chloroplast phosphorylase the values of the $1/S_1$-axis intercepts ranged from approximately 1 to 3, whereas those for the non-chloroplast phosphorylase were one order of magnitude lower. This indicated that the chloroplast phosphorylase had a significantly higher apparent affinity for amylose than the non-chloroplast enzyme.

When the orthophosphate concentration was varied at fixed (saturating and half-saturating) amylose levels, the resulting double reciprocal plots (1/v versus $1/P_i$ concentration) were linear (Fig. 2). Both phosphorylase forms exhibited a similar apparent affinity towards orthophosphate. For the chloroplast enzyme, half-saturating orthophosphate concentrations were 0.9 and 2.1 mmol l^{-1} at saturating and half-saturating amylose levels, respectively. For the non-chloroplast phosphorylase, the corresponding values were 1.1 and 1.7 mmol l^{-1}. Similar values were obtained when amylose was substituted by starch or amylopectin (data not shown).

For glucan phosphorylases from various sources (muscle: Gold et al. 1970; potato: Gold et al. 1971; yeast: Fosset et al. 1971; *Escherichia coli,* Chao et al. 1969), a rapid equilibrium Random Bi Bi mechanism has been proposed. For the determination of kinetic parameters (Table 1) this mechanism was assumed for the two phosphorylases from spinach leaves. According to Cleland (1963; 1970), the following parameters were calculated from the data shown in Fig. 1:

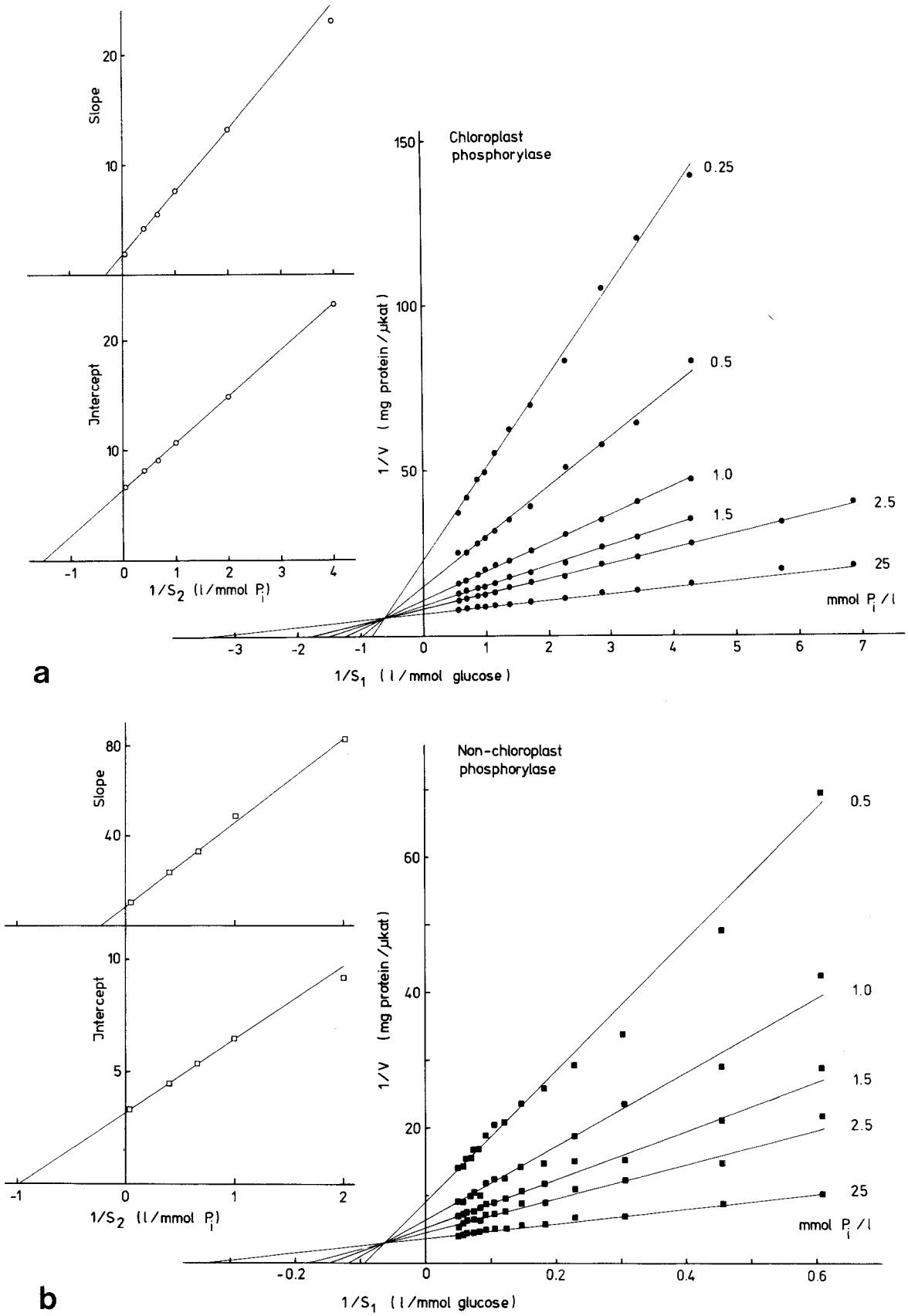

Fig. 1a, b. Phosphorolysis of amylose by the purified chloroplast (**a**) and non-chloroplast (**b**) phosphorylase from spinach leaves. Initial velocities of G1P formation were determined at varying amylose (S_1) and fixed P_i (S_2) concentrations. In a final volume of 1 ml, the assay mixture contained approximately 1.4 µg chloroplast or 0.5 µg non-chloroplast phosphorylase. Right: double reciprocal plot; left: secondary plot (slope or intercept of the 1/v-axis, taken from the Lineweaver-Burk plot, replotted versus $1/S_2$)

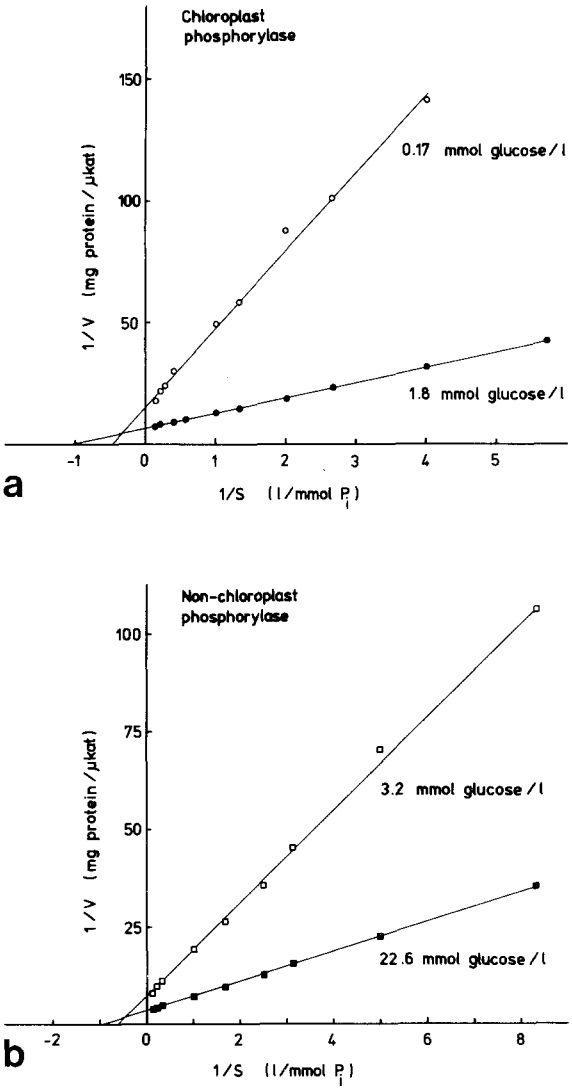

Fig. 2a, b. Phosphorolysis of amylose by purified chloroplast (**a**) and non-chloroplast (**b**) phosphorylase at varying P_i concentrations and fixed (saturating and half-saturating) amylose levels. Double reciprocal plot (1/v versus $1/P_i$)

Table 1. Kinetic parameters of the purified chloroplast and non-chloroplast phosphorylase using amylose as substrate for phosphorolysis. Values were calculated from the data shown in Fig. 1

	Chloroplast Phosphorylase	Non-chloroplast Phosphorylase
K_{iP} (mmol P_i l^{-1})	3.3	4.2
$K_{iAmylose}$ (mmol glucose l^{-1})	1.3	11.5
$K_P = a \cdot K_{iP}$ (mmol P_i l^{-1})	0.7	1
$K_{Amylose} = a \cdot K_{iAmylose}$ (mmol glucose l^{-1})	0.28	2.8
V_{max} (nkat mg^{-1} protein)	156	317
a	0.21	0.24

the apparent Michaelis constants $K_{Amylose}$ and K_P at saturating levels of P_i and amylose, respectively; the apparent dissociation constants K_{iP} and $K_{iAmylose}$ for one substrate in the absence of amylose and P_i, respectively; the velocity of phosphorolysis at saturating P_i and amylose levels, V_{max}, and the interaction factor, a, which describes the effect of the binding of one substrate on the dissociation constant of the other.

The data summarized in Table 1 indicate two significant differences in kinetic properties between the two spinach phosphorylases: the non-chloroplast enzyme had a specific activity approximately twice that of the chloroplast enzyme, and the chloroplast phosphorylase exhibited an approximately 10-fold higher apparent affinity towards amylose both in the presence and in the absence of P_i.

The amylose preparation used in these experiments (Fig. 1 and 2; Table 1) consisted of a mixture of glucans with molecular weights ranging from 1,500–8,000. In order to study phosphorolysis of unbranched glucans in more detail, amylose was substituted by a series of maltodextrins with known chain length and molarity. For both phosphorylases, the minimum chain length required for a significant rate of phosphorolysis was five glucose units per molecule. Using maltose, maltotriose or maltotetraose even at high concentrations (up to 4 mmol l^{-1}), phosphorolysis was either not detectable or occurred at a rate two orders of magnitude lower than the maximum velocity obtained with amylose. Therefore, kinetic measurements were restricted to the maltodextrins G_5–G_{11}. Thin-layer chromatography indicated that the maltodextrins used in these experiments were free of contaminants. Based on the sensitivity of the detection and the amount of substances applied to the plates, impurities of 3% would have been detected. Maltodextrins of the series G_{2n} were completely converted to maltose and traces of glucose by β-amylase treatment, as revealed by thin-layer chromatography (Fig. 3). Under the same conditions, oligosaccharides of the series G_{2n+1} were completely degraded to maltotriose, maltose and traces of glucose. This confirmed that the maltodextrin preparations used in these experiments were unbranched.

Using varying concentrations of these maltodextrins, initial velocities of phosphorolysis were measured at saturating (25 mmol l^{-1}) and approximately half-saturating (1 mmol l^{-1}) P_i levels. All saturation curves were hyperbolic; in the double reciprocal plot straight lines were obtained. For the total maltodextrin series (G_5–G_{11}) the half-saturating glucan concentrations, as determined by the Lineweaver-Burk plot, were plotted versus the chain length of the dextrins added to the assay mixture (Fig. 4). Some observations can be made: As was observed with

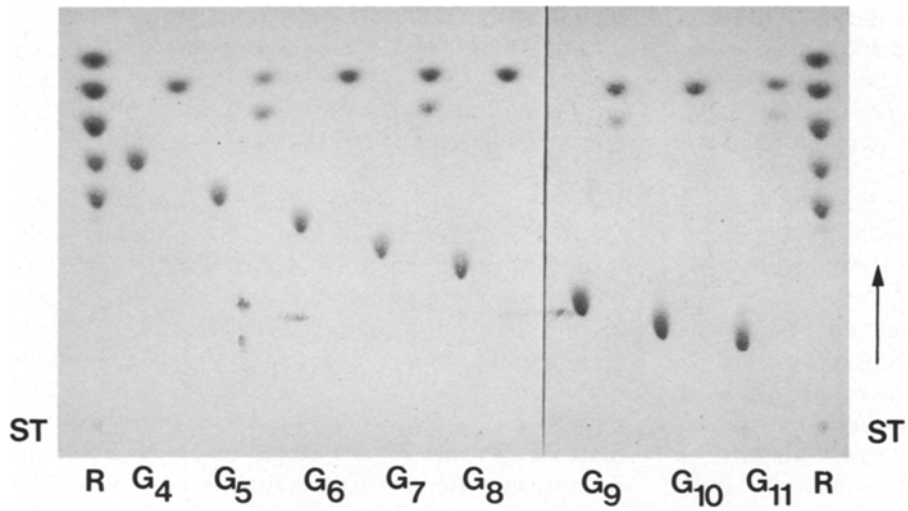

Fig. 3. Thin-layer chromatography of maltodextrins G_4–G_{11}. Approximately 10 µg of each dextrin was applied to the thin layer plate before (left) or after (right) β-amylase treatment (in a final volume of 0.5 ml, 1 mg of each maltodextrin was incubated with 0.3 µkat β-amylase in 10 mmol acetate l^{-1} (pH 5.0) at room temperature for 14 h). Following chromatography, compounds were detected by sulfuric acid treatment. R: mixture of G_1–G_5 as reference. ST: Start. The arrow marks the migration direction

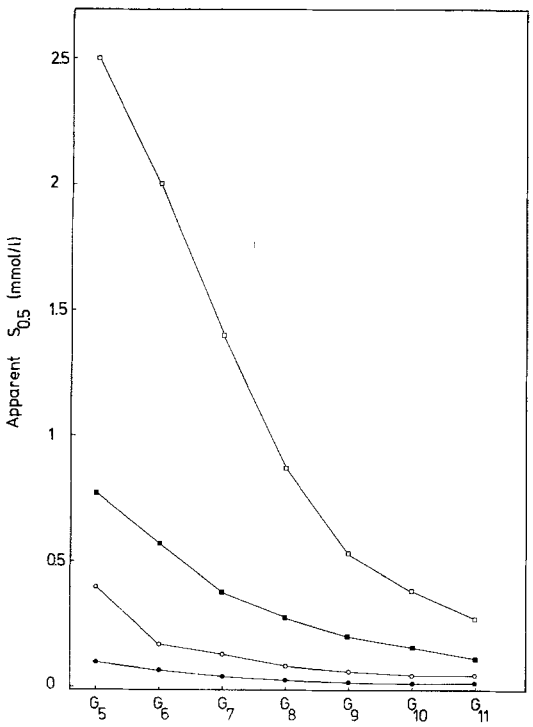

Fig. 4. Phosphorolysis of a series of maltodextrins (G_5–G_{11}) by the chloroplast (○, ●) and non-chloroplast (□, ■) phosphorylase at saturating (25 mmol l^{-1}; closed symbols) and half-saturating (1 mmol l^{-1}; open symbols) P_i concentrations. Apparent half-saturating maltodextrin concentrations (determined by the double-reciprocal plot of the initial reaction velocity versus the dextrin concentration) were plotted versus the chain length of the respective glucan

amylose, both phophorylases exhibited a higher apparent affinity (i.e., the half-saturating dextrin concentrations declined) when the P_i concentration was increased from half-saturating to saturating level. With both phosphorylases, the apparent affinity increased with increasing chain length of the maltodextrins; for the chloroplast phosphorylase half-saturating dextrin concentrations ranged from 100 (G_5) to 16 (G_{11}) µmol l^{-1} at 25 mmol P_i l^{-1} and from 400 to 46 µmol l^{-1} at 1 mmol P_i l^{-1}; the corresponding values for the non-chloroplast enzyme were 770 to 120 and 2,500 to 280 µmol l^{-1} at 25 and 1 mmol P_i l^{-1}, respectively. However, it should be kept in mind that the half-saturating maltodextrin concentrations, as determined at saturating P_i levels, were not necessarily identical with the Michaelis constant for the respective glucan. Phosphorolysis of a dextrin of a given molecular weight yields, as a product, a glucan with a lower degree of polymerization which itself may be used as a substrate for phosphorolysis again. Thus the initially homogenous substrate may become progressively heterogeneous. This effect will be more pronounced at limiting glucan concentrations, but will be less serious if the initial velocity is determined at a very low phosphorylase concentration. Nevertheless, the data shown in Fig. 4 permit the conclusion that the chloroplast phosphorylase, both at saturating and half-saturating P_i levels, had a 7–10-fold higher apparent affinity for low molecular weight dextrins (degree of polymerization $\leqq 11$) than the non-chloroplast enzyme. This difference in apparent affinity was similar to that obtained with the higher molecular weight unbranched glucan, amylose.

Phosphorolytic degradation of the maltodextrins G_5–G_{11} by the two phosphorylases apparently ceased when the dextrin was converted to maltotetraose. This conclusion was reached from experiments in which limiting amounts of maltodextrins were added to the phosphorylase assay; the reaction was followed photometrically until a constant absorbance was reached (Table 2). After cessation of the reaction the amount of glucose 1-phosphate formed did not exceed (*n*-4) moles per mol G_n.

Further evidence for the formation of maltotetraose by the two phosphorylases was obtained in experiments in which the two enzymes were incubated with P_i and various dextrins (with the ommission of the coupled enzyme assay). At intervals, aliquots of the mixture were withdrawn and analyzed by thin-layer chromatography. As an example, the action of the two phosphorylase forms on maltohexaose at 10 mmol P_i l^{-1} is shown (Fig. 5). The product pattern, obtained after 2 h of incubation, remained unchanged when incubation was prolonged up to 8 h. Maltotetraose was the smallest detectable dextrin. However, maltodextrins larger than hexaose (up to G_{12}) also appeared. Elongation of the added dextrin was already detectable after 10-min incubation and at higher P_i levels (50 mmol l^{-1}). Thus, despite the P_i/G1P ratio favoring the phosphorolysis in the overall reaction (Hanes and Maskell 1942), maltodextrin synthesis occured simultaneously. When orthophosphate was replaced by arsenate during incubation, maltohexaose was converted to maltotetraose and glucose (Fig. 5). With arsenolysis, reaction velocity was lower (Katz and Hassid 1950); therefore, a longer incubation time was required in order to achieve an almost complete conversion of maltohexaose.

Effect of maltotetraose on phosphorolysis of glucans. Glucose, maltose, and maltotriose in a final concentration of up to 2 mmol l^{-1} had little or no effect on the phosphorolysis of starch, amylopectin, or amylose by the two phosphorylases (Table 3). However, maltotetraose strongly inhibited chloroplast phosphorylase, but was less effective with the non-chloroplast form (Table 3). This inhibitory effect was studied in more detail using varying starch or amylose concentrations at different fixed maltotetraose levels. The double reciprocal plots of the initial velocities versus starch or amylose concentrations resulted in straight lines with different slopes, but identical intercepts on the 1/v-axis (Fig. 6). The replot of the slope versus the inhibitor concentration was linear. Thus, it appears that maltotetraose is a linear competitive inhibitor with respect to amylose or starch. The inhibition constant, K_i, was 0.1 mmol l^{-1} for both glucans, as determined by the secondary plot. The inhibition of the chloroplast phosphorylase by maltotetraose was also observed when a crude chloroplast extract, as opposed to the purified enzyme, was assayed for activity. In these experiments, saturating levels of P_i (25 mmol l^{-1} and starch (2.55 mmol glucose equiva-

Table 2. Glucose 1-phosphate formation from maltodextrins G_5–G_{11}. The coupled enzyme assay (total volume of 1 ml; P_i concentration 25 mmol l^{-1}) contained 1–1.25 nkat chloroplast or 2.3 nkat non-chloroplast phosphorylase activity. Maltodextrins were added as indicated. Absorbance at 365 nm reached a constant value after 10 min (chloroplast) or 30 min (non-chloroplast phosphorylase). G1P formation was calculated from ΔE_{365}

G_n	Chloroplast phosphorylase			Non-chloroplast phosphorylase		
	Dextrin added	G1P formed		Dextrin added	G1P formed	
	(nmol)	(nmol)	(mol G1P/mol dextrin)	(nmol)	(nmol)	(mol G1P/mol dextrin)
G_5	14.5	14.8	1.02	14.5	13.6	0.94
	32.2	33.3	1.03	14.5	13.6	0.94
G_6	26.3	53.8	2.05	26.3	47	1.79
	52.9	104.5	1.98	26.3	47	1.79
G_7	21.1	62.9	2.98	21.1	57.6	2.73
	37.4	110.6	2.96	21.1	61.4	2.91
G_8	9.09	35.6	3.92	9.09	34.1	3.75
	18.2	72.7	3.99	9.09	33.3	3.66
G_9	8.08	39.4	4.88	8.08	38.6	4.78
	16.6	81.8	4.93	8.08	37.1	4.59
G_{10}	6.91	39.4	5.7	6.91	38.6	5.59
	14.3	81.8	5.72	6.91	37.1	5.37
G_{11}	5.29	34.8	6.58	5.29	34.1	6.45
	10.2	69.7	6.83	5.29	34.8	6.58

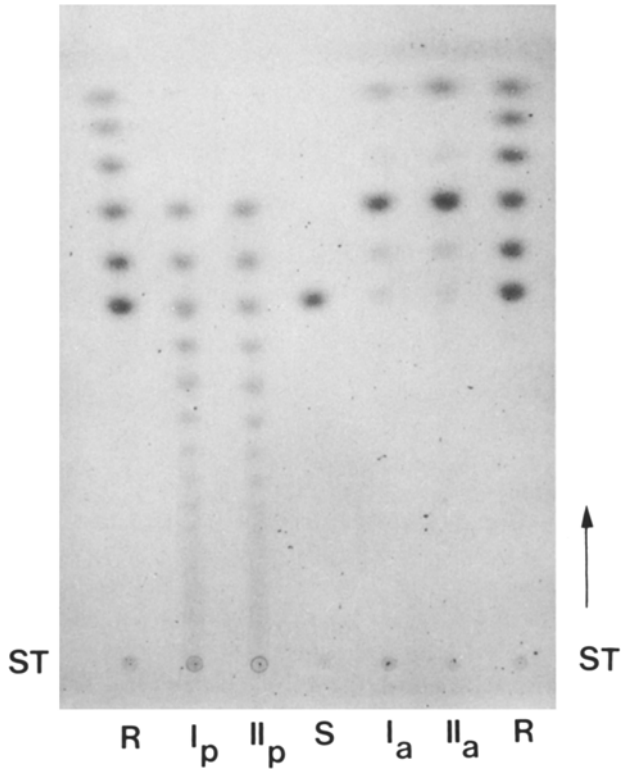

Fig. 5. Analysis of the products of phosphorolysis and arsenolysis of maltohexaose by thin-layer chromatography. Phosphorolysis: in a final volume of 0.4 ml, approximately 1.7 nkat non-chloroplast (I_p) or 0.5 nkat chloroplast (II_p) phosphorylase activity was incubated with 2.5 mmol hexaose l^{-1} and 10 mmol P_i l^{-1} (2 h; 30° C; pH 7.0). Arsenolysis: in a final volume of 0.4 ml, approximately 1.3 nkat non-chloroplast (I_a) or 0.5 nkat chloroplast (II_a) phosphorylase activity was incubated with 2.5 mmol hexaose l^{-1} and 10 mmol arsenate l^{-1} (pH 7.0; 30° C). Incubation was 24 h for the non-chloroplast and 7 h for the chloroplast enzyme, respectively. After incubation, 1 µl of each reaction mixture was applied to the plate. References used were: S (1 µg hexaose), and R (mixture of G_1–G_6; 1 µg of each sugar). ST: Start. The arrow marks the migration direction. Detection of the compounds was with N-(1-naphthyl)ethylenediamine reagent

lents l^{-1}) or amylose (1.75 mmol glucose l^{-1}) were used. With starch as the substrate, phosphorolytic activity of the crude chloroplast extract was 0.7 nkat mg^{-1} chlorophyll; in the presence of 1 and 2 mmol maltotetraose l^{-1} phosphorolytic activities were 0.36 and 0.23 nkat mg^{-1} chlorophyll, respectively. Using amylose as substrate, the corresponding values at 0,1, and 2 mmol maltotetraose l^{-1} were 0.86, 0.45, and 0.3 nkat mg^{-1} chlorophyll, respectively. These results exclude the possibility that the inhibitory effect of maltotetraose might be due to an artifact resulting from the purification of the chloroplast phosphorylase.

Phosphorolysis of branched glucans. Unlike the results obtained with unbranched glucans (amylose and maltodextrins), the non-chloroplast phosphorylase exhibited a significantly higher glucan affinity when branched high molecular weight glucans were offered as substrates. Initial velocities of glucose 1-phosphate

Table 3. Effect of glucose, maltose, maltotriose, and maltotetraose (G_n; final concentration 2 mmol l^{-1}) on the activity of the two phosphorylase forms. Phosphorolytic activity was assayed at saturating P_i (25 mmol l^{-1}) and glucan concentration (starch and amylopectin: 2.7 mmol glucose equivalents l^{-1} for both enzymes; amylose: 15.8 and 2.16 mmol glucose l^{-1} for the non-chloroplast and the chloroplast phosphorylase, respectively). Activities were calculated as percent of the control (no G_n added = 100%)

Glucan	G_n	Non-chloroplast phosphorylase activity (%)	Chloroplast phosphorylase activity (%)
Starch	G_1	100	97.1
	G_2	100	96.8
	G_3	100	92.4
	G_4	105	16.9
Amylopectin	G_1	100	100
	G_2	100	102
	G_3	97	98.2
	G_4	103	12.8
Amylose	G_1	100	98.7
	G_2	94.4	98.8
	G_3	92.5	100
	G_4	84.7	30.8

Table 4. Glucan synthesis by the purified spinach leaf phosphorylases. In a final volume of 1.2 ml, 0.26 nkat chloroplast or non-chloroplast phosphorylase (determined as phosphorolytic activity at saturating starch and P_i levels) was incubated with 50 mmol imidazol-HCl l^{-1}, 50 mmol G1P l^{-1} at pH 7.0. Maltodextrins (G_1–G_7; final concentration 1.6 mmol l^{-1}) or branched glucans (final concentrations for amylopectin, glycogen, and starch were 10.3, 11.2 and 9.5 mmol glucose equivalents l^{-1}, respectively). After 15, 30, 45, and 60 min aliquots of the mixture were withdrawn for P_i determination

Primer	Phosphorylase			
	Chloroplast		Non-chloroplast	
	µmol P_i released after			
	30 min	60 min	30 min	60 min
No primer added	n.d.	n.d.	n.d.	n.d.
G_1, G_2 or G_3	n.d.	n.d.	n.d.	n.d.
G_4	0.93	1.85	0.27	0.55
G_5	1.06	2.04	0.26	0.54
G_6	1.27	2.35	0.42	0.86
G_7	1.26	2.31	0.51	1.02
Amylopectin	0.49	0.89	1.58	3.07
Glycogen	n.d.	0.035	1.57	2.99
Starch	0.6	1.09	1.34	2.6

n.d. = not detectable

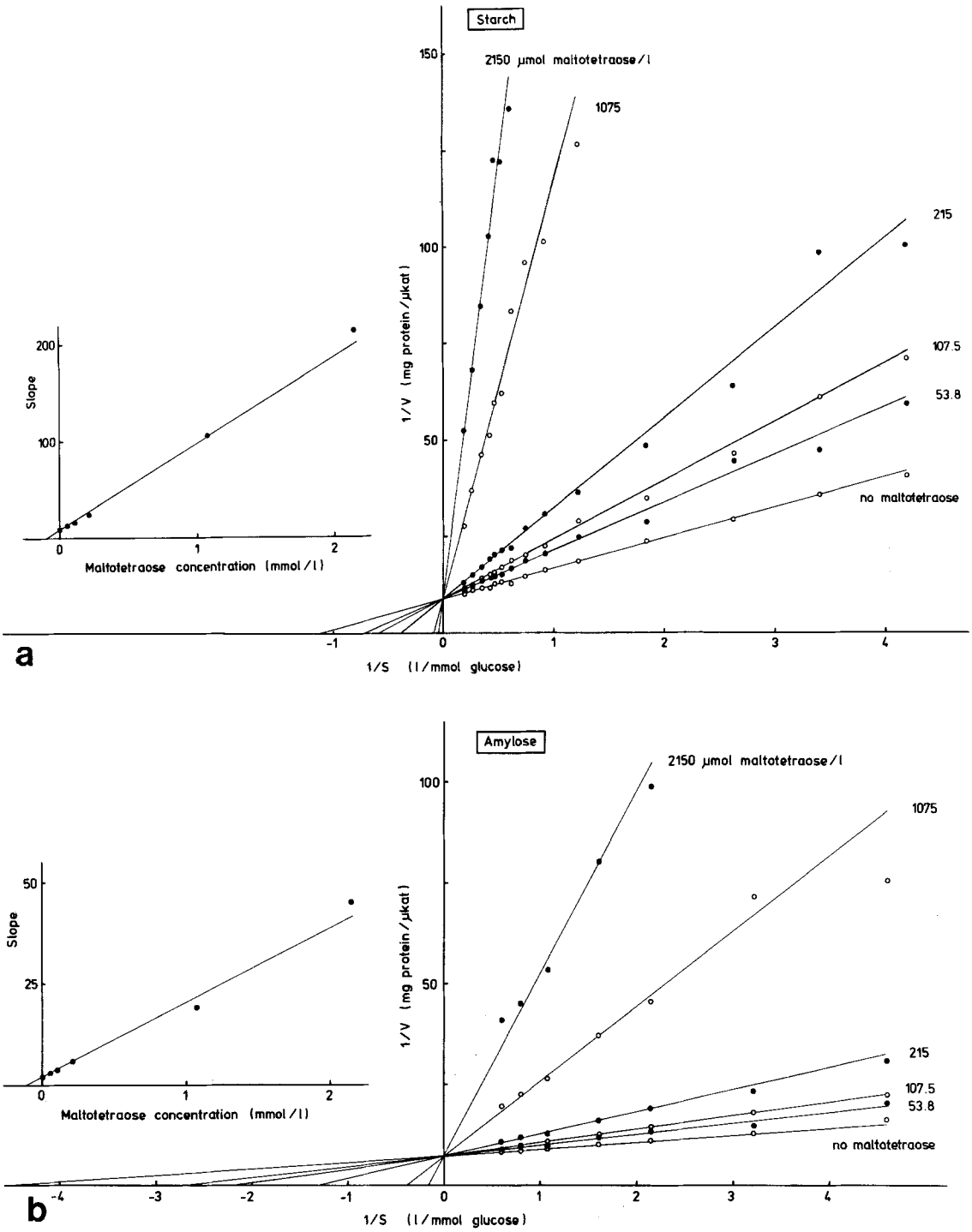

Fig. 6a, b. Phosphorolysis of starch (**a**) and amylose (**b**) by the chloroplast phosphorylase at fixed maltotetraose concentrations. The assay mixture (final volume of 1 ml) contained approximately 1 µg phosphorylase and 25 mmol P_i l^{-1}. Right: double reciprocal plot of the initial velocity versus glucan concentration; left: secondary plot (slope versus inhibitor concentration)

formation were measured with varying concentrations of amylopectin, starch, and glycogen at saturating and half-saturating orthophosphate levels. In all cases hyperbolic saturating curves were obtained (Fig. 7). At 25 mmol P_i l^{-1}, the following half-saturating glucan concentrations for the chloroplast phosphorylase were determined from the Lineweaver-Burk plot: 0.9 mmol glucose equivalents l^{-1} (starch); 0.8 mmol glucose l^{-1} (amylopectin); 30 mmol glucose l^{-1} (glycogen). For the non-chloroplast enzyme the corre-

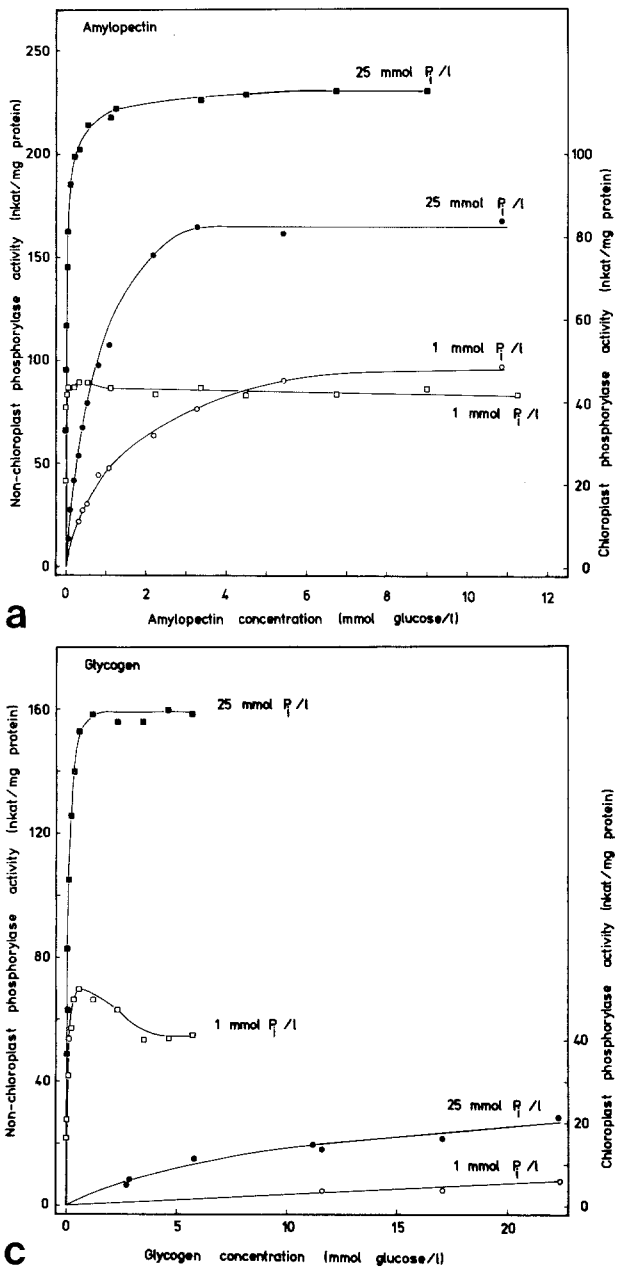

Fig. 7a–c. Phosphorolysis of amylopectin (**a**), starch (**b**), and glycogen (**c**) by the chloroplast (○, ●) and the non-chloroplast (□, ■) phosphorylase at 25 (closed symbols) and 1 mmol P_i l^{-1} (open symbols)

sponding values were: 0.017 mmol glucose l^{-1} (starch); 0.017 mmol glucose l^{-1} (amylopectin); 0.077 mmol glucose l^{-1} (glycogen), thus confirming and extending the preliminary findings obtained with partially purified enzyme preparations (Steup et al. 1980a). At 1 mmol P_i l^{-1}, the half-saturating glucan concentrations for the chloroplast phosphorylase were 2–3 times higher as compared with those at 25 mmol P_i l^{-1}. In contrast, the affinity of the non-chloroplast phosphorylase towards branched glucans apparently did not decrease when the P_i level was lowered to half-saturating concentration. When the non-chloroplast phosphorylase was assayed at 1 mmol P_i l^{-1}, over a wide range of glucan concentrations the initial velocity was unchanged; lowering the glucan concentration shortened the steady-state period (down to less than half a minute) rather than reducing the initial velocity. This effect (which was less pronounced at saturating P_i concentrations, and not detectable at all with unbranched glucans at all P_i concentrations) hampered precize kinetic measurements with the branched glucans at non-saturating orthophosphate concentrations.

Glucan synthesis with branched and unbranched primers. In another series of experiments glucan syn-

thesis by the two phosphorylases was determined using maltodextrins (G_1–G_7), starch, amylopectin, or glycogen as a primer. With both phosphorylases no unprimed glucan synthesis was detected. Maltotetraose was the smallest dextrin capable of priming glucan synthesis. For the chloroplast phosphorylase unbranched low molecular weight glucans (G_4–G_7) were the most effective primers; branched glucans, especially glycogen, were less effective (Table 4). For the non-chloroplast phosphorylase, branched glucans were more effective primers than unbranched dextrins.

Discussion

The two phosphorylase forms, present in spinach leaves, differed significantly in some kinetic properties as revealed by measurements of initial velocity of phosphorolysis. Compared with the chloroplast phosphorylase, the non-chloroplast enzyme had an at least 40-fold higher apparent affinity towards all branched, high molecular weight glucans (starch, amylopectin, glycogen) used in this study. In contrast, the chloroplast phosphorylase exhibited a 7–10-fold higher apparent affinity towards linear glucans, i.e. amylose and maltodextrins (G_5–G_{11}). Supporting evidence for these differences in substrate specifity was derived from experiments in which glucan synthesis was measured (Table 4). With respect to these kinetic properties the chloroplast phosphorylase strongly resembled the type of maltodextrin phosphorylase which has been observed in *Escherichia coli* (Schwartz and Hofnung 1967). Maltotetraose inhibited the chloroplast phosphorylase but was less effective with the non-chloroplast enzyme; this inhibitory effect again points to a similarity between the chloroplast phosphorylase and the maltodextrin phosphorylase (Chao et al. 1969).

On the other hand, the chloroplast and the non-chloroplast phosphorylase forms shared some kinetic properties: They were similar with respect to the pH dependency of the activity, to the apparent affinity for orthophosphate, and to the interaction factor. With both enzymes, phosphorolysis ceased when the glucan was converted to maltotetraose; and a dextrin with a minimum of five glucose units per molecule was required for a significant rate of phosphorolysis. These properties are similar to those described for a sweet corn phosphorylase (Lee and Braun 1973).

Considering the physiological function of the phosphorylase in the glucan metabolism of the plant cell, the data suggest that the chloroplast phosphorylase is not involved in the very first steps of granule degradation. Because of the high apparent affinity towards unbranched glucans with a rather low degree of polymerization, it seems likely that amylase activity (presumably in cooperation with debranching enzymes) preceeds the action of chloroplast phosphorylase, forming the substrate for the latter enzyme. This interpretation is consistent with the observation that the purified chloroplast phosphorylase (and similarly the non-chloroplast enzyme) apparently was unable to form glucose 1-phosphate when starch granules, isolated from intact chloroplasts, were offered as the only glucan (Steup, unpublished results). According to the data shown in Table 2 and Fig. 5, phosphorolytic glucan degradation in the chloroplast would be expected to result in the production of low molecular weight dextrins with a minimum of four glucose units per molecule. At present, it is difficult to estimate the physiological significance of the inhibitory effect of maltotetraose on chloroplast phosphorylase because, to our knowledge, little information is available on the pattern and pool sizes of maltodextrins in chloroplasts. However, it is not inconceivable that maltotetraose plays a role in the coordination of phosphorolytic and amylolytic glucan degradation.

The physiological function of the non-chloroplast phosphorylase is not yet known. However, due to its kinetic properties branched polysaccharides are expected to serve as the physiological substrate rather than linear glucans with a low degree of polymerization.

Finally, it should be mentioned that only rather small differences in the glucan specifity between the two phosphorylases have been described in a recent paper (Preiss et al. 1980). These experiments were performed with a purified non-chloroplast and a partially purified chloroplast phosphorylase preparation. In the cited paper, the kinetic measurements were based on determinations of phosphorolytic glucose 1-phosphate formation after 30-min incubation of the assay mixture. At non-saturating substrate levels, this procedure clearly tends to underestimate the initial phosphorolysis velocities. This especially holds true when the non-chloroplast phosphorylase is assayed with branched glucans as the substrate (cf. page 359).

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