Carbohydrate Research, 227 (1992) 241–255
Elsevier Science Publishers B.V., Amsterdam

(1→4)-α-D-Glucan synthesis by a chloroplastic phosphorylase isolated from spinach leaves is independent of added primer*

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(Received July 17th, 1991; accepted in revised form September 5th, 1991)

ABSTRACT

The chloroplastic phosphorylase from spinach leaf was partially purified using ammonium sulfate fractionation and chromatography on DE-52 and Mono-Q columns. The enzyme was capable of synthesizing (1→4)-α-D-glucan in the absence of added glucan primer, with a lag in time that was shortened by increasing enzyme concentration or by adding bovine serum albumin and/or sodium citrate. The product of the unprimed activity formed a blue complex with iodine–iodide reagent (peak of absorption between 600 and 620 nm) that was insoluble in 5% trichloroacetic acid and in 1% (w/v) KCl in 75:25 methanol–water. Unprimed phosphorylase activity was greatly stimulated by citrate and less by other anions like malate and succinate. Addition of branching enzyme to the assay medium stimulated the unprimed reaction two-fold, or 42-fold if assayed in the presence of 0.1M citrate. In the primed phosphorylase reaction, the presence of 0.8M citrate decreased the K_m for glycogen and amylopectin from 1.8 and 0.17 mg/mL to 0.12 and 0.055 mg/mL, respectively. The phosphorylase fraction (after f.p.l.c.) was found to contain 27 nmol of "anhydroglucose" per mg of protein. It is concluded that the chloroplast phosphorylase from spinach leaves is capable of synthesizing (1→4)-α-D-glucan in the absence of added glucan primer, possibly by elongating an endogenous primer that is associated with the enzyme in a non-covalent fashion.

INTRODUCTION

It is generally believed that ADP-glucose: (1→4)-α-D-glucan 4-α-D-glucosyltransferase (ADP-glucose–starch glucosyltransferase EC 2.4.1.21) is responsible for the formation of the (1→4)-α-D-glucosidic linkages present in starch; hence its common name, starch synthase[1,2]. Starch phosphorylase is also capable of catalyzing the synthesis of (1→4)-α-D-glucosidic linkages *in vitro*, but it is generally accepted that this enzyme is involved in starch breakdown rather than in its synthesis because the ratio of concentrations of orthophosphate (Pi) to D-glucose-1-phosphate (Glc1P) measured *in vivo* would favour phosphorolysis.

Since the first publications reporting the characterization of starch synthase and starch phosphorylase, it was found that they both required exogenous glucan primer to form new α-D-glucosidic bonds, *i.e.*, they could only transfer D-glucose from ADP-glucose or Glc1P, respectively, to a pre-existing glucan primer[1,2]. The same seemed to be

* Dedicated to Professor David Manners.

0008-6215/92/$ 05.00

valid for glycogen synthases of bacteria and mammals[2,3]. Later on, however, a number of reports showed that at least some glycogen synthases from mammals and bacteria can catalyze the synthesis of α-D-glucan in the absence of added primer, *i.e.*, they have "unprimed activity"[3-6]. In plants, unprimed activity has been reported for starch synthases in both potato tuber[7] and spinach leaf[5]. Phosphorylase from rabbit muscle[8] and potato tuber[9] were also found to be capable of synthesizing glucan in the absence of added primer when assayed under suitable conditions.

Several studies on potato tuber were the basis for the hypothesis that, in this storage tissue, a phosphorylase isoform localized in amyloplasts can synthesize (1→4)-α-D-glucan chains on a protein acceptor[9-11]. The protein–glucan so formed would act as a primer for the starch synthase. If initiation of starch biosynthesis *in vivo* followed the pathway proposed, unprimed phosphorylase activity would be expected to be present in all plant tissues that accumulate starch. Two[13-15] (or more[16]) isoforms of phosphorylase are present in leaves of higher plants: one in the chloroplast stroma and another in the cytosolic fraction. The plastidial enzyme was chosen for a detailed study because it is homologous to the potato tuber isoform previously studied[11] and because in the leaf the chloroplast is the site of accumulation of starch. The questions to be answered were the following: Does the chloroplastic phosphorylase have activity in the absence of added primer? Preliminary results[12] showed the presence of a phosphorylase (two isoforms in peas) with unprimed activity like the potato tuber enzyme in the chloroplast stroma of peas, spinach, potato, and *Arabidopsis thaliana*, but other questions remained to be answered. What is the nature of the product of the unprimed activity? Is the endogenous primer a protein? Could the unprimed reaction occur under physiological conditions? Because of the relevance of these questions to the initiation of starch biosynthesis *in vivo*, a variety of methods was employed to answer them. This paper describes the properties of the unprimed activity displayed by the partially purified chloroplastic spinach phosphorylase and its product.

EXPERIMENTAL

Materials. — Maize amylopectin was purchased from Pierce Chemical Company, Rockford, IL; β-amylase from sweet potato and amyloglucosidase from *Aspergillus niger* were purchased from Boehringer–Manheim GmbH.; oyster glycogen and α-amylase from porcine pancreas were purchased from Sigma Chemical Company, St. Louis, MO. Other reagents were of the highest purity available. Purified branching enzymes free of phosphorylase and of other enzymes of starch metabolism were used for stimulation studies. These were the soluble fractions I (41.4 U/mg protein) and IIb (26 U/mg protein) from dent inbred maize prepared as described in ref. 17 with minor modifications (H.P. Guan, unpublished work). Sodium [^{3}H]borohydride (9.09 Ci/mmol) and D-[U-^{14}C]glucose-1-phosphate were obtained from Amersham/Searle, Arlington Heights, IL.

Partial purification of the enzyme. — The chloroplastic phosphorylase was separated from the cytosolic enzyme by a method derived from refs. 14, 15, and 18,

essentially as described in ref. 15, but fractions comprising the second peak of DE-52 were pooled and concentrated by ammonium sulfate precipitation (70% saturation). This fraction (200 mg protein) was dialyzed against 20mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane, pH 6.85 (buffer B) overnight and chromatographed on a Mono-Q column (Pharmacia, HR 16/10). After applying the sample, the column was washed with buffer B, followed by a gradient of KCl in buffer B. The active fractions were pooled and concentrated using an Amicon concentrator. The temperature was kept below 4° throughout the purification.

Preparation of stroma from spinach chloroplasts. — Chloroplasts were isolated from spinach leaves (200 g) by a mechanical method[19] and were then ruptured by osmotic shock by adding 13.2mM sorbitol, 0.4mM KCl, 0.04M EDTA, 0.5mM $MgCl_2$, 2.5mM DTT, and 0.1mM phenylmethanesulfonyl fluoride (PMSF) in 2mM Hepes–KOH, pH 7.9 (50 mL). After centrifugation for 20 min at 20000*g*, the supernatant was concentrated by ammonium sulfate precipitation (70% saturation). The precipitate was resuspended in a small volume of buffer A and dialyzed overnight against the same buffer.

Polyacrylamide gel electrophoresis (PAGE). — Non-denaturating, discontinuous electrophoresis was performed in a Minigel Bio-Rad system (slab gel of 0.75 mm thickness) according to ref. 20, with a monomer concentration of 7.5% in the resolving gel. For the measurement of affinity for glycogen, this carbohydrate was included in the gel in a final concentration of 45 μg/mL. Phosphorylase activity was localized by incubating the gels, after electrophoresis, in a solution containing 20mM Glc1P, 0.1M citrate–NaOH, pH 5.3, and, for the detection of primed activity, one also containing 0.08% soluble starch. After incubation for 2–3 h, the gels were stained by transferring them to a solution containing 0.2% KI and 0.02% I_2 in 0.2M buffer acetate–NaOH, pH 4.8. The presence of phosphorylase activity was detected as blue band(s) on the gel. For detection of amylase activity, the gels were incubated for 3 h in a solution containing 0.2% soluble starch, 0.1M citrate–NaOH, pH 5.3, and then they were stained with the iodine reagent. Amylase activity was detected as lighter bands in the dark blue background.

Electrophoresis in the presence of SDS was performed using the system of Laemmli[21] (7% total monomer in the separation gel, 0.75 mm thickness). The samples, containing 5M urea, 10% 2-mercaptoethanol, 2% SDS, 10% sucrose, and 0.5 mg/mL bromophenol blue, were heated for 2 min in boiling water prior to electrophoresis.

PAGE of amylose and the phosphorylase product was carried out in the presence of SDS and urea following the method of Ziegler[22] (7.5% total monomer, 0.75 mm thickness).

In all types of PAGE, protein bands were detected using Coomassie Brilliant Blue[23], and (1→4)-α-D-glucan was detected using the iodine–iodide reagent as described above.

Enzyme assays. — *Phosphorylase assay A.* Phosphorolytic activity was determined by the coupled assay[24] using oyster glycogen (1.5 mg).

Phosphorylase assay B (primed activity). The transfer of D-[^{14}C]glucose into methanol-insoluble material in the presence of exogenous primer was measured in reaction mixtures containing 1050 nmol D-[U-^{14}C]glucose-1-P, specific activity 53 c.p.m./nmol, 5 μmol 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.2, 150 μg glycogen, and enzyme in a total volume of 50 μL. Reactions were incubated at 37° and were stopped by heating in boiling water. Carrier glycogen (0.9 mg) was added, followed by the addition of a 75% methanol solution containing 1% KCl (2 mL). The washed precipitate was redissolved in water (1 mL), and an aliquot (0.5 mL) was counted with Safety Solve (5 mL, Research Products Laboratory, Mount Prospect, IL) using a Packard Tri-Carb model 1600 CA scintillation counter (Packard Instrument Co., Downers Grove, IL).

Phosphorylase assay C (unprimed phosphorylase activity). Transfer of D-[^{14}C]glucose in the absence of added primer was determined as in assay B, except that glycogen was replaced with bovine serum albumin (125 μg). Reactions were stopped in one of the following ways: *i*) Methanol precipitation, as in phosphorylase assay B. *ii*) Trichloroacetic acid (TCA) precipitation. The reaction was stopped by adding 5% TCA (1 mL). The washed precipitate was redissolved with 0.04N NaOH (1 mL), heated, and counted as in assay B. *iii*) Measurement of the iodine–carbohydrate complex. Unlabelled Glc1P was used in this assay. The incubation mixture (50 μL) was mixed with dimethyl sulfoxide (Me_2SO, 30 μL), followed by the iodine reagent (1 mL). The iodine reagent was 0.05% KI and 0.005% I_2 in 0.05M buffer acetate–NaOH, pH 4.8. Absorbance at 610 nm, or the absorbance spectrum (440–740 nm) of the D-glucan–I_2 complex was measured immediately after addition of the iodine reagent.

Protein determination. — Protein was measured using the BCA reagent[25] (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as standard.

Measurement of "anhydroglucose" in the enzyme preparation. — An aliquot of the enzyme fraction was exhaustively dialyzed against water, made 2N in HCl, and hydrolyzed for 1 h at 100°. The samples were evaporated under vacuum, dissolved in water, and centrifuged. The supernatant was neutralized, and D-glucose was determined by measuring NADPH formation when aliquots were incubated in reaction mixtures containing hexokinase and glucose-6-phosphate dehydrogenase[26]. The final amount was corrected for loss of the internal glucose standard (about 20%). A similar procedure was carried out with bovine serum albumin.

Treatment of the enzyme with amyloglucosidase. — Chloroplast phosphorylase (6 μg, f.p.l.c. fraction) was incubated with amyloglucosidase (10 μg) in 0.1M MES, pH 6.0 for 45 min at 37°, in a total volume of 5 μL. After incubation, the sample was electrophoresed under non-denaturating conditions. Phosphorylase and amylase activities were localized in the gel as described above.

Product analysis and enzyme digestion. — The reaction mixture of unprimed phosphorylase as in assay C was scaled up. The TCA precipitate was centrifuged, washed, and redissolved with 0.01N NaOH (0.4 mL), heated gently for 1 min at 70°, neutralized with 0.1N HCl, and adjusted to 0.5 mL. Aliquots (8 μL) containing 6000 c.p.m. of D-[^{14}C]glucose were taken and added immediately to the incubation mixtures

containing the corresponding hydrolytic enzyme in order to minimize retrogradation of the product before the enzymes could act on it. The enzymes were (a) β-amylase (140 units) in 0.02mM Tris acetate buffer, pH 8.0; (b) α-amylase (27 units) in 0.02M phosphate buffer, pH 7.0, plus 0.1mM KCl; (c) amyloglucosidase (0.7 units) in 0.02mM acetate buffer, pH 4.0. Incubations were carried out for 24 h at 37°. The reaction products were chromatographed on Whatman No. 1 paper for 40 h in 6:4:3 butanol–pyridine–water. Radioactive areas were excised and counted.

Periodate oxidation and NaB[^{3}H]$_4$ reduction of chloroplast phosphorylase. — Periodate oxidation of the chloroplast phosphorylase (f.p.l.c. fraction, 0.75 mg protein), followed by reduction with NaB[^{3}H]$_4$ was carried out using the procedure of Yasuda *et al.*[27] with minor modifications. The same procedure was followed for commercial ovalbumin, except that 1.7 mg of protein was used. Aliquots of the protein samples obtained by periodate oxidation and sodium borohydride reduction were mixed with sample buffer (final concentrations: 2% SDS, 5M urea, 10% 2-mercaptoethanol), heated for 3 min in boiling water, and applied to disc-gels. Two gels were run for each protein: one was stained for protein, and the other was sliced into 3-mm slices which were placed into scintillation vials, dried, and digested with a mixture of 99:1 (v/v) 30% H_2O_2: conc. NH_4OH (0.4 mL) for 16 h at 37° before counting.

RESULTS

Presence of unprimed phosphorylase activity in the stroma of spinach chloroplasts. — Electrophoresis under non-denaturing conditions was used to follow the two isoforms present in the leaf extract[13–15,28] throughout the purification procedure and to confirm the identity of the enzymes by comparing their electrophoretic behaviour with the phosphorylase present in the chloroplastic stroma fraction. One band with R_F 0.33 and having both primed and unprimed phosphorylase activity was present in the stromal fraction. The affinity for glycogen was examined by including 45 μg/mL of glycogen in the polyacrylamide gel[14]. Migration of the band was not affected by the presence of this branched polysaccharide in the gel.

Presence of two isoforms with unprimed phosphorylase activity in spinach leaf. — Using non-denaturing PAGE, two isoforms with R_F values of 0.33 and 0.36 and displaying both primed and unprimed activity were detected in both the crude extract and in the ammonium sulfate fractions. The faster band was retarded by the presence of glycogen in the gel, with the R_F decreasing from 0.36 to 0.04, indicating that the enzyme had relatively high affinity for glycogen[13,14]. The mobility of the slow band was unchanged. The migration of the two bands, and their response to the inclusion of glycogen in the gel, supported the identification of the isoforms, *i.e.*, the slower band corresponds to the chloroplastic phosphorylase and the faster one to the cytosolic form.

Partial purification of the chloroplastic phosphorylase (phosphorylase II). — Purification of the chloroplastic phosphorylase to homogeneity has been achieved using affinity chromatography on Sepharose-bound starch especially modified to achieve binding of the enzyme[14]. However, the objective of this work was to study the unprimed

activity, and it was considered essential to avoid any steps that might introduce glucan primer into the preparation. For this reason, the procedure described by Steup[14] was not used. We substituted instead an ammonium sulfate fractionation and chromatography on DE-52 column, followed by another ion-exchange chromatography on a Mono-Q column.

To follow purification throughout the first stages, assay A in the phosphorolysis direction was used because of the presence of interfering activities, which included amylases. The ammonium sulfate step decreased amylases to the extent that unprimed phosphorylase activity could be detected using assay C. Alternatively, phosphorylase activity, both primed and unprimed, could be detected, even in the crude extract, using non-denaturing PAGE as described above. Chromatography on DE-52 separated both isoforms, and it was found that peak I contained the faster moving phosphorylase (phosphorylase I^{14}) with a high affinity for glycogen, and peak II (phosphorylase II^{14}) contained the slower migrating form with a low affinity for the branched polysaccharide[14,15].

Although the chromatography on DE-52 separated the two phosphorylases, the Mono-Q step was required to separate the chloroplastic phosphorylase from the branching enzyme activity present in peak II (see below). The phosphorylase peak appeared at 0.29M KCl. Using assay B, it was determined that the f.p.l.c. step had purified the phosphorylase 24-fold and that the yield was 54%. The presence of branching activity in the peak II (DE-52 fraction) was demonstrated by using the iodine–iodide reagent in the measurement of the unprimed activity. The peak wavelength of the product–iodine complex was 610 nm for the f.p.l.c. fraction (blue colour) and was 572 nm (purple) for the product of peak II. The effect of branching activity in the determination of unprimed activity is reported below under "Effect of activators".

The final preparation (*i.e.*, after f.p.l.c.) was assayed for glucan associated with the protein and was found to contain 27.2 nmol of "anhydroglucose"* units per mg of protein (about 0.17 nmol in a 50 μL volume of assay mix when 6.25 μg were used, or 3.4 nmol per mL of assay medium).

Properties of the unprimed activity. — The properties of the final preparation were examined with respect to the unprimed reaction. Fig. 1A shows the effect of time on the formation of the methanol-insoluble product. In Fig. 1B the reaction is followed by the absorbance at 610 nm of the complex between the product and the iodine reagent. There was a lag with time, and after this period was over, the reaction was linear. No difference in the incorporation of D-[^{14}C]glucose could be detected using precipitation with 5% TCA or with methanol (not illustrated). The product of the unprimed reaction formed a blue complex with the iodine–iodide reagent. In the experiment illustrated in Fig. 1B, the peak of absorption of the complex increased gradually from 590 nm (at 20 min of incubation) to 613 nm (60 min incubation); routinely, absorbance at 610 nm was used to measure the amount of product formed. As shown in Figs. 1A and 1B, the kinetics of the

* "Anhydroglucose" (sometimes termed "polyglucose") is defined as that non-dialyzable material that produces D-glucose upon acid hydrolysis. A mol. wt. of 162 was assumed.

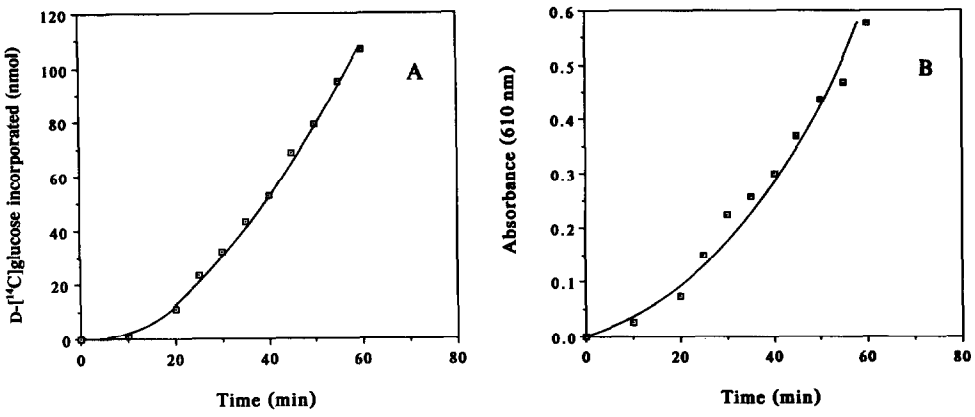

Fig. 1. Effect of the time of incubation on the formation of the unprimed product by the chloroplast phosphorylase from spinach leaf. Unprimed phosphorylase activity was measured by the incorporation of D-[^{14}C]glucose into material that was precipitated by 5% TCA (A) or by the formation of a product which formed a blue complex with the iodine–iodide reagent (B). The conditions were as in assay C, and the assay mixture contained 0.04M D-[^{14}C]glucose-1-P, 2.5 mg/mL BSA, 0.1M sodium citrate (pH 6.2), and 5 μg of enzyme.

reaction was similar regardless of the method used for the measurement of the product, and a ΔA_{610} of 0.1 corresponded to the incorporation of 21 nmol D-[^{14}C]glucose into a product that was precipitated by 5% TCA or 75% methanol. Because of the good correspondence between the three methods of detecting unprimed activity, the formation of the complex with the iodine reagent was used in a number of experiments described below.

Optimal pH for activity. Effect of activators. — The unprimed activity of the phosphorylase II has a sharp optimal pH, around pH 6.2. The activity was much higher when buffer citrate was used, and the incubation times needed to determine activity with MES and HEPES buffers were much longer than with citrate. No activity was detected using Tris/HCl buffer between pH 7.0 and 8.0.

The K_m for Glc1P, determined from a Michaelis–Menten plot, was found to be 1.9mM and was measured in the presence of 0.1M citrate because of the very long lag with time of the reaction at the lower Glc1P concentrations. For the Lineweaver–Burk plot, the rates used were determined after the lag.

BSA increased the rate of the unprimed reaction and shortened the lag in time, and its effect was further increased by the presence of citrate. It is worth noting that the commercial preparation of BSA used was found to contain 1.3 nmol "anhydroglucose" per mg protein (0.16 nmol in a 50-μL volume of assay medium, or 3.2 nmol per mL of assay medium). The unprimed activity was also stimulated by other polyvalent anions like malate, succinate, and EDTA, but to a lesser extent than by citrate. In assay conditions in which 0.1M citrate brought about a 5.3-fold stimulation, the same concentrations of malate and succinate resulted in stimulation of 3.2 and 2.6 times the control, respectively.

Even when assayed in the presence of 0.5M citrate and 2 mg/mL BSA, the rate of the unprimed reaction was much lower (5%) than the rate of the primed reaction (assay B).

At low enzyme concentrations, the lag with time lengthened. At the lowest concentration of enzyme used (1 μg in the assay), no unprimed activity was detected. The unprimed reaction was not linear with respect to added enzyme.

The unprimed reaction was strongly activated by branching enzymes from maize kernel. Addition of branching enzyme (fraction I from maize endosperm[17]) to the assay medium shortened the lag in time, and the heat-inactivated enzyme did not affect the kinetics of the reaction (Fig. 2A). Fig. 2B illustrates the saturation curve for branching enzyme fraction I and shows that the heat-inactivated enzyme had no detectable effect on the reaction at the concentrations used. Table I compares the effect of two different branching enzymes from maize, fractions I and II, in their interactions with the unprimed phosphorylase and citrate. These experiments were performed at pH 7.0 in order to evaluate the rate of the unprimed reaction near physiological conditions.

It is worth noting that absorbance of the complex between the product and the iodine reagent (at 610 nm) could be used to measure the rate of the reaction because the peak wavelength was not affected by the presence of branching within the reaction times and at the concentrations of branching enzyme used. Peak wavelength increased slightly with incubation time. When longer incubation times (4 h) were used, the presence of branching enzyme shifted the peak wavelength from 625 to 603 nm.

Fraction I, at the concentration used in these experiments, stimulated the unprimed reaction to such an extent that the additional presence of citrate did not affect the reaction much further. Conversely, fraction IIb did not stimulate the unprimed reaction in the absence of citrate, except to the extent that heat-inactivated enzyme

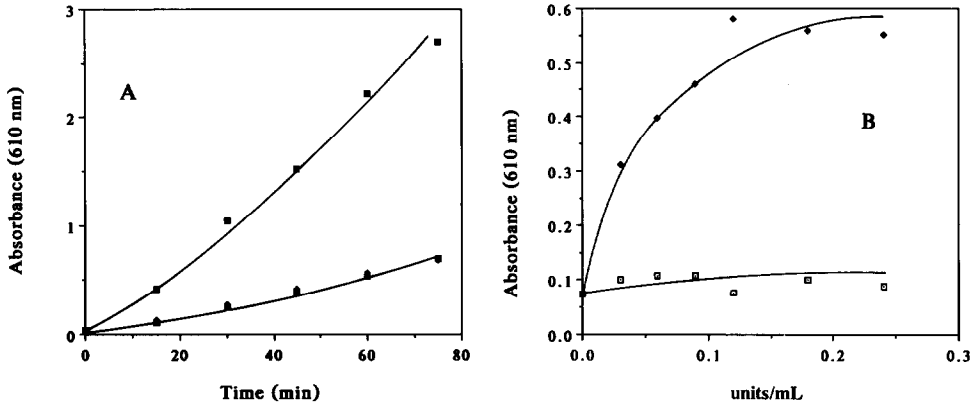

Fig. 2. The effect of purified branching enzyme (fraction I of maize kernel) on the citrate-stimulated reaction catalyzed by phosphorylase II in the absence of added primer. Heat-inactivated branching enzyme had no effect at the concentrations used in these experiments. A: Time course in the presence (■) of branching enzyme; in its absence (◆); or with added-heat-inactivated branching enzyme (□). B: Saturation curve for the stimulation by branching enzyme (◆); with heat-inactivated enzyme (□).

TABLE I

Effect of citrate and branching enzymes on unprimed phosphorylase activity

Addition	*Absorbance (610 nm)*	*Stimulation (×-fold)*
None	0.068	1
+ 0.1M citrate	0.279	4.1
+ Branching enzyme I[a] (heat-inactivated)	0.131	1.9
+ Branching enzyme I	2.844	42
+ 0.1M citrate + Branching enzyme I (heat-inactivated)	0.441	6.5
+ 0.1M citrate + Branching enzyme I	3.285	48.3
+ Branching enzyme II[a] (heat-inactivated)	0.197	2.9
+ Branching enzyme II	0.211	3.1
+ 0.1M citrate, + Branching enzyme II (heat-inactivated)	0.516	7.6
+ 0.1M citrate + Branching enzyme II	1.217	17.9

[a]0.015 units of the fraction of branching enzyme indicated in the table were used. Incubation time, 1 h. Enzyme: 3.3 μg, BSA 2.5 mg/mL.

activated it. It is likely that some carbohydrate is associated with this fraction, possibly some of it introduced during the purification procedure (ref. 17, modified by H. P. Guan, unpublished work). In the presence of citrate, fraction I did activate the unprimed reaction, but the contribution of the heat-inactivated component also increased.

In the conditions employed in Table I, the fraction I enzyme activated very little after heat inactivation (but not at lower concentrations, see Fig. 2), but citrate was shown to increase this effect.

Effect of the treatment of the enzymatic fraction with amyloglucosidase. — The unprimed activities displayed by some enzymes have proved to be sensitive to the action of carbohydrate-degrading enzymes, indicating that glucans associated with the enzyme preparation were acting as endogenous primers. To examine this possibility, the f.p.l.c. fraction was incubated with amyloglucosidase. Taking advantage of the fact that the electrophoretic mobilities of the chloroplastic phosphorylase and amyloglucosidase are different[10], the incubation mixture and appropriate controls were electrophoresed, and it was found that the unprimed activity of the phosphorylase (measured in the gel) was unaffected. In the conditions used for non-denaturing PAGE, the amylases appear as two main bands with R_Fs 0.43 and 0.58, and a minor band with R_F 0.31.

Periodate oxidation and NaB[^{3}H]$_4$ reduction of partly purified phosphorylase. — The behaviour of the partly purified phosphorylase after its oxidation with periodate and subsequent reduction with NaB[^{3}H]$_4$ was very different from that of the tritiated ovalbumin. The recovery of radioactivity from the gels was about 45% for ovalbumin and only 1% for the phosphorylase fraction. Of the radioactivity recovered in the case the ovoalbumin gel, 95% was associated with the protein, with some radioactivity remaining in the stacking gel. For the phosphorylase fraction, the main band which corresponds to the phosphorylase and with mol. wt. 105 000, and the minor, contam-

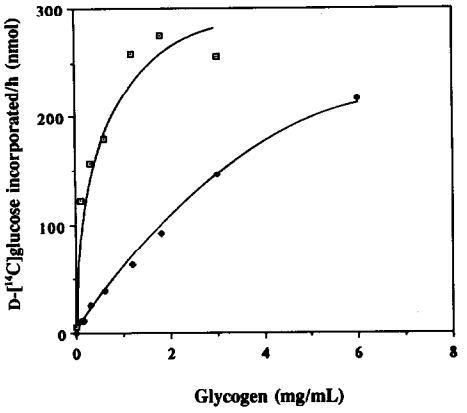

Fig. 3. Effect of sodium citrate on the affinity of chloroplast phosphorylase for oyster glycogen: (□) 0.8M citrate; (◆) no citrate.

inating bands (with mol. wt. 98, 67, 58, 53, 45 and 28 kdaltons) had some radioactivity associated with them, but it was clear that most of the radioactivity did not enter into the resolving gel, and that only a minor part remained in the stacking gel. These results suggest that the treatment had labelled carbohydrate present in the enzyme preparation, and that the tritiated glucan was not bound to the protein in a covalent manner.

Effect of citrate on the phosphorylase affinity for glycogen and amylopectin. — In the primed phosphorylase reaction, the presence of 0.8M citrate decreased the K_m for glycogen and amylopectin from 1.8 and 0.17 mg/mL to 0.12 and 0.055 mg/mL, respectively. The rate of the reaction at substrate saturation was not affected by citrate in the case of glycogen (Fig. 3), but it decreased to about half for amylopectin. This decrease in rate was probably due to retrogradation of the elongated amylopectin. Indeed, it was difficult to determine a rate at the concentrations of amylopectin near saturation because in the presence of citrate the rate decreased with time. At the end of a 30-min incubation, a precipitate could be observed that required boiling for 2 min in 40% Me_2SO for solubilization.

Characteristics of the unprimed product. — Aliquots of the TCA-precipitated product of the unprimed reaction were treated with the hydrolytic enzymes α-amylase, β-amylase, and amyloglucosidase. After enzyme treatment and separation of the products by paper chromatography, the distribution of radioactivity remaining at the origin and co-chromatographing with marker glucose, maltose, or maltotriose was estimated and the data are summarized in Table II. These data, taken together with the information regarding the complex formed by the product with the iodine–iodide complex, indicate that the product is a higly linear (1→4)-α-D-glucan with a degree of polymerization of 70 to >400 glucosyl units[29]. The small percentage of radioactivity remaining in the origin in the case of the treatment with β-amylse, rather than indicating the presence of (1→6)-α-D-linkages, could be attributed to some retrogradation of the linear glucan occuring despite of the great care taken to redissolve it completely in alkaline medium and add the amylolytic enzymes immediately after neutralization.

TABLE II

Distribution of radioactivity in separated products after enzymatic digestion of [^{14}C]glucan formed by chloroplastic phosphorylase

Treatment	*Radioactivity in*[a] *(%)*			
	Origin	*Maltotriose*	*Maltose*	*Glucose*
β-Amylase	8	2	81	10
α-Amylase	3	5	42	50
Amyloglucosidase	0	0	0	100

[a]Values represent the percentage of recovered radioactivity remaining at the origin or co-chromatographing with the marker sugars indicated. Overall recoveries of radioactivity were 70–80% of the counts present prior to digestion.

The unprimed products formed by the chloroplastic and cytosolic phosphorylase from spinach leaf were subjected to electrophoresis in the presence of SDS and urea[11]. Part of the glucan (as detected with the iodine–iodide reagent) migrated towards the anode in two bands of mobility equivalent to that of proteins of mol. wt. 90 000–100 000. Corn and potato amyloses and amylopectins commercially prepared behaved in a similar manner. Very faint protein bands were detected using Coomassie Brilliant Blue, but they did not co-migrate with the glucan. Glycogen from rabbit liver and from oyster (commercially obtained) did not migrate into the gel.

DISCUSSION

Purification of the chloroplastic phosphorylase from spinach leaves has been previously reported by several authors who also studied its properties. The affinity of the enzyme for glucans has also been studied, and it was found that the chloroplastic isoform has a much lower affinity for branched polysaccharides than the cytosolic form[13]. The data reported above show that the affinity for branched polysaccharides increased significantly if citrate was included in the assay. The K_m value for Glc1P in the unprimed reaction as determined here, 1.9mM, is lower than that obtained by Shimomura *et al.*[13] (3.3mM) for the primed reaction. Preiss *et al.*[28] reported a broad pH–activity curve for the chloroplast phosphorylase. However, the pH curves in ref. 13 show a sharp optimum in both directions of assay, *i.e.*, phosphorolysis and glucan synthesis with an optimal pH of 6.1. For the unprimed synthesis, both the shape of the curve and the optimal pH of the unprimed reaction resemble the results of Shimomura *et al.*[13] Together, these data indicate that the unprimed reaction (studied here) and the primed reaction (studied by several authors) are very similar, except for the lag observed in the absence of primer.

Affinity chromatography has been carried out on starch or on a starch derivative as part of the purification procedure[13–15]. It is worth noting that Shimomura *et al.*[13] reported unprimed activity (15–35% of the activity in the presence of saturating primer)

for the cytosolic form (which contained sugar in the range of 1%) but no obvious activity in the absence of primer was detected for the chloroplastic phosphorylase. In the present study, peak I from DE-52 (cytosolic enzyme) displayed unprimed activity with no obvious lag (not illustrated), confirming the findings reported in ref. 13, but peak II (and the f.p.l.c. fraction purified from it) had unprimed activity. The difference possibly stems from the different assay conditions. Indeed, the unprimed activity may go undetected if the assay mixture contains low concentrations of enzyme, particularly in the absence of BSA as assayed in ref. 14.

From the enzymes capable of unprimed glucan synthesis, several have been found to contain glucan in such amounts that the assay medium contained the following amounts of "anhydroglucose": starch synthase from maize kernel, 1–25 μg/mL[30], 1 μg/mL[31]; starch synthase from spinach leaf, 0.1 μg/mL[32]; potato tuber phosphorylase (plastidial form) 6–25 μg/mL[10]. The spinach cytosolic phosphorylase contained 1% D-glucose as measured by the phenol–sulfuric acid method[13]. From the bacterial enzymes, the glycogen synthase from *Escherichia coli* contained 25 μg of "anhydroglucose" per unit enzyme[33]. In comparison, the chloroplastic spinach phosphorylase contains relatively little "anhydroglucose" (see Results section). The unprimed activity was unchanged by incubation with amyloglucosidase, as was the case with the potato tuber phosphorylase[10] and starch synthase[34], suggesting that the glucan primer is not accessible to the hydrolytic enzyme. Conversely, the unprimed activity of the glycogen synthase of *Escherichia coli* was shown to be sensitive to treatment with amylolytic enzymes[35].

BSA is included in the assay mediums of all the unprimed activities mentioned above. Although it activates the unprimed reaction, the nature of this activation is unknown. As shown above, the commercial preparation seems to contain very small amounts of primer glucan, and although this component may be significant when compared with the amounts of glucan contributed by the phosphorylase preparation, it would be negligible in other unprimed reactions in which the enzyme preparation contains large amounts of "anhydroglucose".

The unprimed activities of the starch synthases I from maize kernel[31] and from spinach leaf[32], and that of the bacterial glycogen synthase[33], have been shown to be activated by citrate. In all of these, citrate also decreased the K_m for primer by a large factor, to the extent that in the case of the starch synthase I from maize kernel, Pollock and Preiss[31] reported that it was not possible to determine a K_m for amylopectin and glycogen in the presence of 0.5M citrate because under these conditions the enzyme catalyzed the primed and unprimed reactions at similar rates. From the above it can be seen that the potato phosphorylase is the only enzyme in which citrate has been shown not to activate the unprimed reaction, but the enzyme had been only partially purified; indeed, the enzymatic fraction probably contained some branching and amylase activity which could have affected the formation of the product that could be precipitated by 5% TCA. The nature of the activation by citrate (and by other tricarboxylic acids) of the unprimed reactions is not known. Although citrate can favour aggregation of some enzymes, this property in itself does not explain the activation of the unprimed activ-

ities. It is possible that citrate may form a complex with the enzyme and the glucan substrate, as suggested by the accelerated retrogradation observed when assaying the phosphorylase with amylopectin as a primer or even in the unprimed reaction in the presence of branching enzyme. The activation by citrate could be partly due to a stimulation of branching enzyme present in the preparation, as is the case for the starch synthase. However, it is unlikely that the f.p.l.c. fraction contained significant amounts of branching, if any, as the peak wavelength of the product–iodine complex is high (> 600 nm as compared with values of 585–600 nm for the starch synthase[31]), indicating that the product is linear. Whatever the mechanism of activation of citrate, its relevance in the understanding of the unprimed reactions is that when it stimulates the unprimed activity in question, it also increases the affinity of the enzymes for glycogen and amylopectin.

Sivak and Tandecarz[12] isolated chloroplasts from spinach and three other species that accummulate starch in leaves — *Arabidopsis thaliana*, peas, and potato — and found that the phosphorylases present had unprimed activity, suggesting that this is a widespread characteristic of chloroplastic phosphorylases. At present, there is no reason to believe that the acceptor of the unprimed chloroplastic phosphorylase is a protein, nor that the glucan formed is bound to a protein. The unprimed product of the chloroplastic phosphorylase migrates in SDS–PAGE as if it were attached to a protein moiety, and, although this behaviour has been interpreted in the past as suggestive of a proteoglucan nature of the unprimed product of the potato phosphorylase[10,11], the tendency of long (1→4)-α-D-glucan chains to associate with SDS[36,37] would allow the product to migrate electrophoretically even if it were not attached to a protein moiety. Other criteria for the identification of an unprimed product as a proteoglucan have been tested and discussed elswhere, *i.e.*, precipitation with TCA, hydrolysis with diluted acid, and extensive treatment with proteases, and they have been shown to be unsuitable for the identification of an unprimed product of phosphorylases or glycogen or starch synthases as proteoglucans[3,10,11,33].

Although the results of the present and other studies seriously question whether *de novo* synthesis of carbohydrates really occurs in some of the systems examined, initiation as a glucosyl–protein remains a viable hypothesis. Tandecarz and Cardini[38] have described a system which comprises at least two enzymatic reactions in which proplastid membranes from potato tuber glycosylate a membrane protein using UDP-Glc to form a glucoprotein which, in turn, is used as an acceptor for a long chain of glucoses sequentially added in a (1→4)-α-D-bond using either ADP-Glc or UDP-Glc as donors. This system has been further characterized, and one of the enzymes has been purified[39,40].

CONCLUSIONS

It seems that most (if not all) of the enzymes capable of elongating glucans can display activity independent of added primer when assayed under suitable conditions. The question that remains to be answered is whether conditions *in vivo* allow these

activities to occur. Citrate and other intermediates of the tricarboxylic cycle are known to be present in the chloroplast stroma. Branching enzyme is present in the stroma of chloroplasts and amyloplasts. The ratio of D-Glc1P to Pi is generally believed to favour phosphorolysis, but compartmentation of Pi, which allows limitation of photosynthesis in some circumstances[41], might sometimes allow the reaction to proceed in the synthesis direction.

ACKNOWLEDGMENTS

This research was supported by a grant to M. N. S. from the Research Excellence Funds from the State of Michigan and the O.E.C.D., and grants to Dr. Jack Preiss from the U.S.D.A/D.O.E./N.S.F. Plant Science Center Program (88-37271-3964). In its initial stages, this research was performed at the Institute for Photosynthesis Research, Sheffield, and supported by the Agricultural and Food Research Council, U.K. I wish to thank Dr. H. P. Guan for providing purified branching enzyme fractions from maize, and Drs. Juana Tandecarz and Jack Preiss for advice and criticism.

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