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Starch biosynthesis: mechanism for the elongation of starch chains

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Abstract

Starch granules from eight diverse plant sources all had active starch synthases and branching enzymes inside the granules. The enzymes synthesized both amylose and amylopectin from ADPGlc. Pulsing of the granules with ADP-[^{14}C]Glc gave synthesis of starch that on reduction and glucoamylase hydrolysis gave ^{14}C-labeled D-glucitol. The pulsed label could be chased by nonlabeled ADPGlc to give a significant decrease of ^{14}C-label in D-glucitol. Evidence further indicated that the synthase forms a high-energy covalent complex with D-glucose and the growing starch chain, and that the D-glucopyranosyl group is added to the reducing end of the growing starch chain by a two-site insertion mechanism. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The energy for the biosynthesis of polysaccharides is provided by the attachment of the monomer to an activating group through a high energy, labile linkage. Two distinct mechanisms have been proposed for the polymerization of the monomer units.[1] The first mechanism involves the successive addition of activated monomer units to the nonreducing end of a preformed primer chain with the release of the activator group (Reaction A in Fig. 1). The second mechanism involves an activated monomer unit that is added to the reducing end of a second activated monomer in a transglycosylation reaction that displaces the activator group of the second monomer. The chain is then elongated by the successive additions of monomer units to the reducing end of the growing chain by a transglycosylation two-site insertion mechanism (Reaction B in Fig. 1). A modification of the first mechanism has been proposed for the biosynthesis of glycogen and starch in which an enzyme transfers D-glucose residues from a nucleotide glucosyl diphosphate to an endogenous protein acceptor (glycogenin or amylogenin) in the absence of a primer.[2–5] It was proposed that a limited number (6–10) of D-glucose residues are added to the protein acceptor, giving a maltodextrin chain attached to the protein. This maltodextrin chain then acts as a classical primer to which a synthase catalyzes the addition of D-glucose residues from the activated monomer to the nonreducing end of the primer attached to the protein (Reaction C in Fig. 1).

The nonreducing end primer mechanism for the elongation of a polysaccharide chain arose from the studies of Cori and Cori[6] and Swanson and Cori[7] with muscle glycogen phosphorylase and Hanes[8] with potato starch phosphorylase. It was found that phosphorylase from the respective sources could elongate glycogen and starch chains by the transfer of D-glucose from α-D-glucopyranosyl phosphate (α-Glc-1-P) to the nonreducing ends of the glucans. For a relatively long period, it was believed that phosphorylase was the synthetic enzyme for glycogen and starch biosynthesis. The synthesis required a preformed polysaccharide or maltodextrin chain, called a primer. It was later shown, however, that phosphorylase was a degradative enzyme, rather than a synthetic enzyme, that catalyzed the reaction of inorganic phosphate (P_i) with the nonreduc-

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ing end glucose residue of the polysaccharide chain to form α-Glc-1-P as in the following reaction.[9]

$$P_i + \underset{\text{starch chain}}{\text{G-G-G-G-G-}\cdots} \underset{\text{synthetic}}{\overset{\text{degradative}}{\rightleftharpoons}} \underset{\alpha\text{-Glc-1-P}}{\text{G-P}} + \underset{\text{so-called primer}}{\text{G-G-G-G-}\cdots}$$

The synthetic reaction is, thus, the reverse of the degradative reaction, and would, therefore, also have to require a nonreducing end of a preformed polysaccharide chain for the synthetic reaction to occur as shown in the above reaction. This preformed or required polysaccharide chain is the origin of the so-called required primer, nonreducing end mechanism for starch biosynthesis.

When the phosphorylase reaction was conducted in the synthetic direction, that is, by starting with α-Glc-1-P and starch or glycogen primer, the reaction added glucose to the nonreducing ends of the starch or glycogen primer and would then rapidly slow down and stop after the addition of several D-glucose residues. It was shown that the equilibrium ratio of P_i to α-Glc-1-P at pH 6.8 is 3.6[10] and the concentration of P_i in plant and animal tissue is many fold higher than α-Glc-1-P,[11] making the reaction in the synthetic direction very improbable. So, both for the in vitro and the in vivo reactions, the conditions for the synthesis of starch by phosphorylase are quite unfavorable. The nonreducing end, primer-dependent mechanism, based on the phosphorylase reaction, had been assumed for the biosynthesis of all polysaccharides from 1940 to 1970, and it has been the working hypothesis for the biosynthesis of starch for over 60 years, from 1940 to the present.

In 1961, Leloir et al.[12,13] discovered a new pathway for the biosynthesis of starch in which nucleotide glucosyl diphosphate (UDPGlc and ADPGlc) was the glucosyl donor for starch biosynthesis by the enzyme, starch synthase. It was and has been assumed that starch synthase was a primer-dependent enzyme, similar to phosphorylase, which transferred D-glucose from UDPGlc or ADPGlc to the nonreducing end glucose residue of a primer chain.[13–17]

In 1967, *Salmonella* O-antigen polysaccharide was shown to be biosynthesized by the transfer of an activated repeating tetrasaccharide unit to the reducing end of another activated tetrasaccharide unit, giving elongation by an insertion mechanism[18,19] (Fig. 1(B)). The reaction was not dependent on a preformed oligosaccharide or polysaccharide chain or primer. This same type of elongation mechanism was subsequently shown for several polysaccharides: namely, bacterial cell-wall polysaccharide, murein;[20] *Leuconostoc mesenteroides* B-512F dextran;[21] *Streptococcus mutans* alternating comb

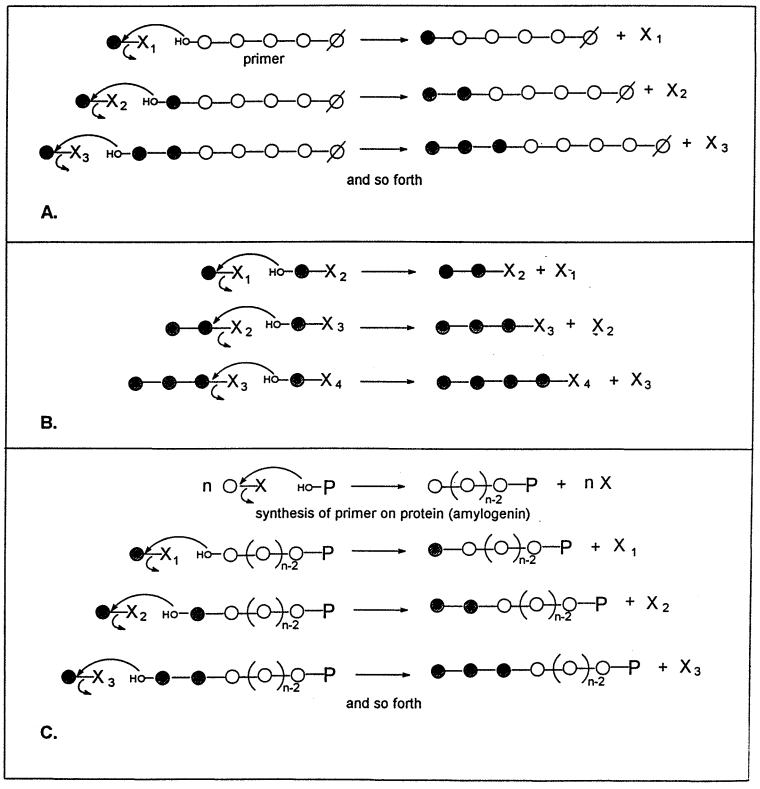

Fig. 1. Possible mechanisms for starch chain biosynthesis: (A) Synthesis by the addition of monomer units to the nonreducing end of a preformed primer polysaccharide or oligosaccharide chain. (B) Synthesis by addition of monomer units to the reducing end by insertion. (C) Synthesis of a primer oligosaccharide on a protein and then elongation by addition of monomers to the nonreducing end of the primer oligosaccharide.

dextran and mutan;[22] *Xanthanomonas campestris* xanthan;[23] acetan;[24] and *Acetobacter xylinum* cellulose.[25]

It previously was reported that starch granules contain active synthetic enzymes that synthesize starch chains from ADPGlc.[12,13] In the present study, we pulsed starch granules with ADP-[^{14}C]Glc and then chased with nonlabeled ADPGlc. The resulting data indicate that D-glucopyranosyl residues are transferred from ADPGlc to the reducing end of a growing starch chain, which is attached to starch synthase by a labile covalent linkage and that the elongation is most probably by a two-site, insertion mechanism.

2. Experimental

Materials.—Adenosine α-D-glucopyranosyl 5′-diphosphate (ADPGlc) and *Aspergillus niger* glucoamylase (type A 3042) were obtained from Sigma Chemical Co. (St. Louis, MO). The glucoamylase (5 mL, 6100 IU) was dialyzed at 4 °C against two 1 L 50 mM pyridine–acetate buffer (pH 5.2) dialyzates for 15 h each to remove D-glucose.

ADP-[^{14}C]Glc (242 mCi/mmol) was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Rice, taro, and potatoes were purchased from local supermarkets. Hard red spring wheat berries, hulled barley, and rye berries were purchased from a local organic food store. Maize seeds, that had been harvested after 20 days growth, had starch with the usual 22:78 ratio of amylose to amylopectin, were obtained from Dr Martha James (Department of Biochemistry, Biophysics, and Molecular Biology at Iowa State University). Waxy maize seeds, containing starch with 100% amylopectin, were obtained from Dr Mark Campbell (Truman State University, Kirksville, MO). The liquid scintillation cocktail contained 5.0 g PPO and 0.1 g POPOP in 1.0 L of toluene. All other chemicals were of the highest grade commercially available and were used without further treatment.

Starch isolation.—Potato and taro starches were isolated and purified according to a standard procedure used for potato starch.[26] Rice and maize starches were prepared from seeds using the procedures established for maize starches.[27] Wheat, barley, and rye starches were prepared as described for wheat starch.[28] All of the isolated starches were air dried at ambient temperature and then stored at 4 °C.

Measurement of starch synthase activity.—The synthesizing activity of each of the varieties of starch granules was assayed by determining the amount of radioactivity that was transferred from ADP-[^{14}C]Glc into water-insoluble starch granules. Starch granules (100 mg) were suspended in 1.0 mL of 0.1 mM EDTA and 4 mM glycine buffer (pH 8.4). The reaction was initiated by adding 2 μmol (1 μCi) of ADP-[^{14}C]Glc. The reaction was terminated by centrifuging and washing the starch granules five times with 1 mL of water each to obtain background radioactivity. This removed unreacted ADPGlc from the granules and terminated the reaction. The granules were then treated three times with 1 mL of anhyd acetone to remove water. Small amounts of acetone were removed from the granules by pulling a vacuum for 1 min. The acetone-dried granules were weighed and the radioactivity incorporated into the granules was measured by adding the granules to a toluene scintillation cocktail, followed by heterogeneous liquid scintillation counting.

Pulsing and chasing of starch granules with ADPGlc.—

1. Starch granules (3 g) were suspended in 15 mL of 0.1 mM EDTA and 4 mM glycine buffer (pH 8.4).
2. ADP-[^{14}C]Glc (1.5 μCi, no carrier, giving 6.2 nmol ADPGlc) was added, giving 0.42 μM ADPGlc, and the reaction was allowed to proceed at 20 °C for 30 or 1 min.
3. The 30 min pulsed starch granules were centrifuged 3 min and washed five times with 15 mL of water each to give background counts. For the 1 min pulse, the starch was filtered and washed five times directly on the filter.
4. The starch granules were then suspended in 15 mL of water and a 5 mL sample of the suspension (1 g of starch) was taken for the pulse sample.
5. The remaining starch suspension was centrifuged and the granules (2 g) were suspended in 9 mL of buffer to which 1 mL of 200 μmol/mL of nonlabeled ADPGlc, giving 20 μmol of ADPGlc/mL (20 mM) for the chase reaction at 20 °C.
6. The chase reaction was sampled at 30 and 120 min by taking 5 mL (~ 1 g of starch). The reaction was stopped by centrifuging and washing the granules five times with 5 mL of water for each sample.
7. The pulse and chase granules were then each suspended in 5 mL of 0.01 M HCl (pH 2) and incubated at 50 °C for 90 min.
8. Each of the samples were centrifuged and washed twice with 5 mL of water and then suspended in 8 mL of water to which 0.5 mL of pyridine and 1.0 mL of $NaBH_4$ (10 mg/mL) were added, and the samples were incubated at 70 °C for 1 h.
9. The resulting gels were broken apart by vortexing for 1–2 min with 1 mL of glass beads. Each sample was then slowly poured into 200 mL of boiling water with stirring and heated at 100 °C for 10 min to destroy any excess borohydride and completely gelatinize and solubilize the starch.
10. The solutions were cooled and diluted to 250 mL in a volumetric flask from which 10 mL were removed from the pulse sample, concentrated to 1 mL, and added to Whatman 3 MM paper (2 × 5 cm), dried, and placed into 10 mL of toluene

scintillation cocktail and counted in a liquid scintillation spectrometer to determine the amount of ^{14}C incorporated into the starch.

11. Acetic acid (5 M) was added to the starch solution in the volumetric flask to give pH 5.2; 20 IU of glucoamylase (one IU equals the amount of enzyme that will produce 1 μmol of D-glucose from waxy maize starch at pH 5.2 and 37 °C in 1 min) was added to the solubilized starch and hydrolysis was allowed to proceed at 37 °C for 15–16 h.
12. Commercial baker's yeast, *Saccharomyces cerevisiae*, (1 g) was added to each sample and fermentation was allowed to occur at 37 °C for 15–16 h to remove D-glucose.
13. The yeast was removed by centrifugation and filtration, and the solution was concentrated to ~10 mL. A TLC was made (two ascents of 17:3 MeCN–water on Whatman K5, 5 × 10 cm plate) to determine if the majority of the D-glucose had been removed from the digest.
14. The 10 mL volume was then concentrated to 1 mL, and 500 μL was added along 15 cm of the top and center of two Whatman 3 MM papers (23 × 54 cm) for descending paper chromatography,[29] using 8:1:1:1 volume proportions of $MeNO_2$–AcOH–EtOH–water saturated boric acid for 15–16 h.[30]
15. Standards of D-glucitol, D-glucose, and maltitol were added to both sides of the chromatogram and developed by the $AgNO_3$ technique.[30] These were used to locate the potential radioactive compounds from the starch hydrolyzates on the chromatogram.
16. After development, the papers were sectioned (3 × 15 cm) where the radioactive D-glucitol was located, thoroughly dried, and placed into 10 mL of toluene scintillation cocktail and counted in a liquid scintillation spectrometer.

Separation of labeled amylose and amylopectin from pulsed samples.—Starch granules (1 g) were pulsed by suspending them in 5.0 mL of 0.1 mM EDTA, 4 mM glycine (pH 8.4), containing 0.5 μCi ADP-[^{14}C]Glc. The reaction was allowed to proceed at 20 °C for 30 min. The starch granules were centrifuged and washed five times with 5.0 mL of water to give background radioactivity.

The amylose and amylopectin components were separated by precipitation of the amylose with thymol.[31] The pulsed granules were dissolved by stirring them in 10 mL of 1 M NaOH for 1 h, followed by neutralization with 1 M HCl to pH 7, and 350 μL of EtOH, containing 10% (w/v) thymol, was added and the solution stirred at 20 °C for 15–17 h. The resulting precipitate (amylose–thymol complex) was centrifuged and the supernatant, containing amylopectin, was removed. The thymol complex was washed three times with 5 mL of water. Two volumes of EtOH were added to the supernatant to precipitate the amylopectin, which was removed by centrifugation. The two precipitates, amylose–thymol complex and amylopectin, were each treated with 5 mL of acetone three times to remove water and thymol and then with 5 mL of anhyd EtOH. The EtOH was removed and the precipitates were then dried in a vacuum oven at 40 °C for 15 h. The precipitates were weighed and 50 mg were added to 10 mL of toluene liquid scintillation cocktail and the radioactivity measured by heterogeneous liquid scintillation counting.

Testing the effect of pH 2 treatment after pulsing.—Starches (2.5 g) were suspended in 12.5 mL of 4 mM glycine and 0.1 mM EDTA buffer (pH 8.4); 1.25 μCi (5.2 nmol) of ADP[^{14}C]Glc were added and the reaction allowed to proceed for 30 min; two 6.0 mL aliquots (~1 g of starch each) were filtered and the starch washed five times with 10 mL of water each; 1 g was weighed from the two aliquots. One of the samples of starch was suspended in 5.0 mL of 0.01 M HCl, giving a pH of 2.0, and incubated for 90 min at 50 °C. The starch was centrifuged and washed twice with 5 mL of water. The two starches, one treated at pH 2 and the other not treated, were each suspended in 8.0 mL of water and 0.5 mL of pyridine and 1.0 mL of $NaBH_4$ (10 mg/mL) were added to each and they were incubated at 70 °C for 1 h. The starches were then treated as per steps (9) and (11) through (16) of the pulse and chase protocol.

3. Results and discussion

The eight varieties of starch granules used in the studies came from widely diverse plant sources, representing seed and tuber starches. They had widely differing granule morphologies, representing large, medium, small, and very small sized granules, containing differing amounts of amylose and amylopectin components. All eight of the starch granules contained active starch synthesizing enzymes, synthases and branching enzymes, that incorporated D-glucose into starch from ADPGlc (Table 1). They synthesized both the amylose and the amylopectin components (Table 2). The formation of amylose from waxy maize was unexpected and after a long reaction time, the amylose disappeared into the amylopectin component.

Pulsing of the eight varieties of starch granules with ADP[^{14}C]Glc gave the formation of ^{14}C-labeled D-glucitol after the reduction of the reducing end of the synthesized starch chains (Table 3). Significantly long chains were synthesized in the pulse reactions that had average d.p. values, ranging from 827 to 127 D-glucose residues, depending on the particular variety of starch. When the pulsed samples were chased with nonlabeled ADPGlc, the amount of ^{14}C-label in D-glucitol was

Table 1
Starch synthase activity in starch granules reacting with 20 mM ADPGlc

Starch	Starch synthase activity (nmol glucose/h/100 mg of starch granules)
Maize	272
Wheat	202
Barley	126
Rye	122
Taro	84
Rice	44
Potato	39
Waxy maize	16

Table 2
Amount of pulsed label from ADP-[^{14}C]Glc incorporated into the amylose and amylopectin components

Starch [a]	Fraction	Cpm [b]	%
Maize	amylopectin	324,545	74.9
	amylose	108,760	25.1
Waxy maize	amylopectin	139,442	92.4
	amylose	11,469	7.6
Potato	amylopectin	70,738	86.8
	amylose	10,921	13.4

[a] The pulsed starch granules were gelatinized and the amylose precipitated with thymol, removed, and the amylopectin precipitated by the addition of 2 vols of ethanol.

[b] Each of the samples were counted for 10 min or 10,000 cpm, whichever came first and the background count subtracted.

significantly decreased (Table 3). The pulse and chase experiments were performed for a minimum of two–three times and a maximum of five times for each of the starches (maize 5 ×, potato 4 ×, waxy maize 3 ×, rice 3 ×, rye 3 ×, wheat 2 ×, barley 2 ×, and taro 2 ×), giving essentially the same pattern of labeled D-glucitol in the pulse that was chased by nonlabeled ADPGlc. The formation of ^{14}C-labeled D-glucitol in the pulse reaction and its decrease in the chase reaction shows that the elongation of the starch chains takes place by the addition of D-glucose from ADPGlc to the reducing end of the growing starch chain.

In previous studies on the biosynthesis of dextran, it was found that dextran was tightly associated with the synthesizing enzyme, dextransucrase. The dextran could be released from the enzyme by treatment at pH 2, 95 °C.[21,22] Cellulose was shown to be covalently linked to a lipid pyrophosphate intermediate during biosynthesis and could be released from the lipid pyrophosphate by a pH 2, 100 °C treatment.[32,33] We, therefore, postulated that in the biosynthesis of starch, the synthase most probably makes a similar covalent linkage with the growing starch chains and consequently treated the pulsed and chased starch granules at pH 2, 50 °C for 90 min to release newly synthesized starch chains from the enzyme. The fact that the ^{14}C-label in the D-glucitol was not decreased to zero or values close to zero by the chase reactions (Table 3) for most of the starches, suggested that the covalent linkage between the starch chains and the enzyme was labile and that some of the chains were being hydrolyzed from the enzyme during the course of the synthesis. This would then explain why the ^{14}C-label was not being completely chased from the reducing end of the starch chain and hence from the D-glucitol.

Table 3
^{14}C-Labeled D-glucitol obtained from 30 min pulse with ADP-[^{14}C]Glc and chase with ADPGlc

Starch [a]	D-Glucitol pulse (cpm) [b]	D-Glucitol 30 min chase (cpm) [b]	D-Glucitol 120 min chase (cpm) [b]	Total (cpm) [c]	Average d.p. [d]
Maize	524	314	258	433,305	827
Waxy maize	348	284	218	151,675	436
Taro	305	89	52	141,008	462
Rice	228	136	130	106,550	467
Wheat	175	156	104	83,275	476
Potato	124	91	85	65,025	524
Barley	196	35	30	24,803	127
Rye	50	16	16	22,058	441

[a] The different starches were pulsed and chased for a minimum of 2–3 times and a maximum of 5 times: maize 5 ×, potato 4 ×, waxy maize 3 ×, rice 3 ×, rye 3 ×, wheat 2 ×, barley 2 ×, and taro 2 ×. The results were similar for the repeat experiments and the data presented in the table are those of the last experiment.

[b] Each sample was counted for 10 min or 10,000 counts, whichever came first, and the background count subtracted.

[c] Total cpm in D-glucitol and D-glucose.

[d] d.p., average degree of polymerization of pulsed starch chains = total cpm/cpm of D-glucitol.

Table 4
D-Glucitol obtained from pulse of starch granules with pH 2 treatment and without pH 2 treatment

Starch	w pH 2 (cpm) [a]	w/o pH 2 (cpm) [a]	% of chains released [b]
Maize	575	300	52
Waxy maize	419	329	79
Wheat	456	246	54
Potato	229	124	54

[a] Each of the samples were counted for 10 min and the background count subtracted. Each of the experiments were preformed twice, giving consistent results of a significantly greater amount of label in the pH 2 treated pulsed granules than in the untreated pulsed granules.

[b] Percent, (cpm D-glucitol w/o pH 2/cpm D-glucitol w pH 2) × 100, of the starch chains released from the enzyme by hydrolysis during the course of the pulse reaction, indicating their relative lability of their attachment to starch synthase.

To test this hypothesis and to simultaneously demonstrate that the starch chains were indeed covalently linked to the starch synthase during synthesis, we compared the amount of ^{14}C-label in D-glucitol from pulsed granules that were treated at pH 2, 50 °C for 90 min with the amount of label in D-glucitol from pulsed granules that were not treated at pH 2, 50 °C for 90 min (Table 4). In all cases, there was more labeled D-glucitol obtained when the pulsed granules were treated at pH 2 than there was when they were not treated at pH 2, indicating that the starch chains are covalently linked to starch synthase during synthesis. However, there was also a significant amount of labeled D-glucitol obtained when the granules were not treated at pH 2, indicating that the linkage between the starch chain and the enzyme was relatively labile and that some of the chains were being released from starch synthase during the course of the 30 min pulse reaction.

The pH 2-treatment (0.01 M H^+) at 50 °C for 90 min was determined insufficient to hydrolyze the α-(1→4) glycosidic linkages of the starch chains and give reducing ends that could be reduced to give labeled D-glucitol. This was shown by treating maltose at pH 2, 50 °C for 90 min, followed by analysis of D-glucose by the micro glucose oxidase assay.[34] No D-glucose was found where 0.001% hydrolysis should have been detected, indicating that the α-(1→4) glycosidic linkage is stable under the conditions of the pH 2 treatment. Thus, pH 2 treatment must hydrolyze a labile linkage between the enzyme (synthase) and the reducing end of the starch chain as indicated by the data of Table 4. The most probable nature of this linkage is that of a carboxyl-acetal. The differences in the degree of susceptibility to the hydrolysis of the linkage between the enzyme and the different starches may reflect differences in the structures of the active sites of the enzymes or differences in the environments in which the synthases are located within the different starch granules, or both. The labile, high-energy linkage between the starch chain and the enzyme is necessary for the synthesis of the α-(1→4) linkage in the starch chain.

To study the hydrolysis of the linkage between the synthase and the reducing end of the growing starch chain further, we pulsed some of the starch granules for 1 min instead of 30 min. The results show (Table 5) that labeled D-glucitol is still formed, although in a lesser amount as expected than the 30 min pulse, and that the labeled D-glucitol was chased to a relatively low value, confirming that the covalent linkages between the enzyme and the starch chains are relatively labile and some of them are released in the relatively long reaction time of 30 min, giving the synthesis of new chains.

We considered other possibilities for the formation of labeled D-glucitol that was observed in the pulse reaction. For example, it might be that D-glucitol was being formed by the reduction of D-glucose that was produced from the action of glucoamylase by reaction with some residual borohydride that had not been destroyed by boiling the starch solution for 10 min (step (9) in the pulse–chase protocol). To determine whether the borohydride had been completely destroyed, a control experiment was performed in which maltose was added to the mixture after the boiling step and was incubated at 37 °C for 15 h. No maltitol was formed, as judged by TLC, confirming that the borohydride had been completely destroyed by boiling for 10 min, and D-glucitol was not formed by residual borohydride reduction of

Table 5
^{14}C-Labeled D-glucitol obtained from a 1 min pulse with ADP-[^{14}C]Glc and chase with ADPGlc

Starch	Pulse 1 min (cpm) [a]	Chase 30 min (cpm) [a]	Chase 120 min (cpm) [a]	Total (cpm) [b]	Average d.p. [c]
Maize	74	21	13	23,500	318
Waxy maize	78	41	26	56,185	720
Taro	52	29	15	28,125	541

[a] Each of the samples were counted for 10 min and the background count subtracted.

[b] Total cpm in D-glucitol and D-glucose.

[c] d.p., average degree of polymerization of pulsed starch chains = total cpm/cpm of D-glucitol.

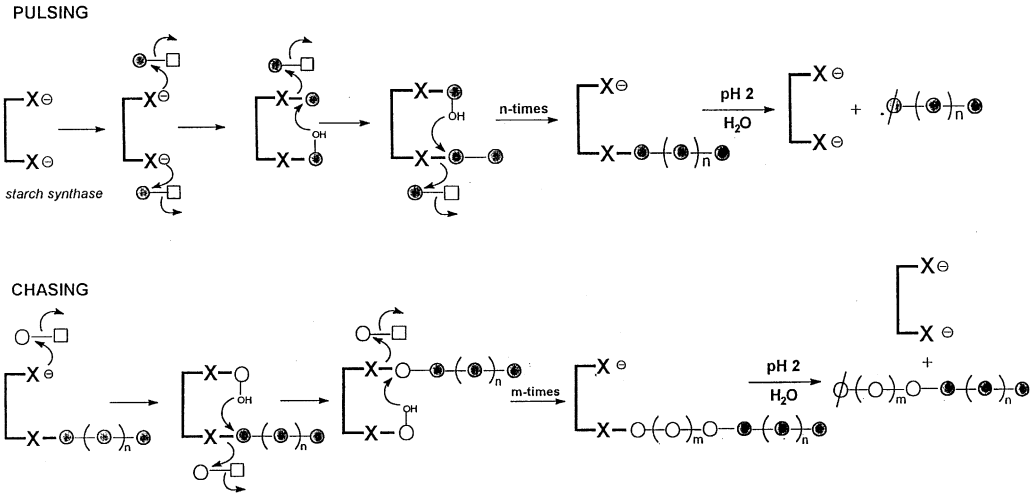

Fig. 2. Pulse and chase reactions of starch synthase, showing the addition of D-glucose to the reducing end of a growing chain by a two-site, insertion mechanism. Shaded circles represent ^{14}C-labeled D-glucose; open circles represent nonlabeled D-glucose; the circles attached to a square represents glucose attached to ADP (ADPGlc); the negatively charged X represents a nucleophile at the active-site of starch synthase. The reducing end of the starch chain is on the left-hand side and the nonreducing end is on the right-hand side; a reducing group is indicated by a circle with a slash through it.

D-glucose. Another possibility for the formation of labeled D-glucitol from a pulse reaction, might be the hydrolysis of a labeled chain by amylase or debranching enzyme, or by branching enzyme in which the starch chain is hydrolyzed from the enzyme and not transferred to another chain. These enzyme-catalyzed, hydrolytic reactions could indeed give labeled reducing ends that could be reduced to give labeled D-glucitol, but there would be no possibility for this labeled D-glucitol to be chased by nonlabeled ADPGlc as was observed. Further, if some hydrolytic enzyme was producing reducing ends, there should not have been any change in the amount of D-glucitol formed from the pulse reaction with the pH 2 treatment and from the pulse reaction without the pH 2 treatment. The last possibility that we considered was that the yeast was forming labeled D-glucitol from D-glucose in the reverse reaction of a D-glucitol dehydrogenase reaction during the fermentation of the D-glucose. This was tested by a control experiment in which an amount of ^{14}C-labeled D-glucose, equivalent to the amount of ^{14}C-labeled D-glucose in the pulse and chase experiments, was fermented by *S. cerevisiae*. No labeled D-glucitol was found. Further, it again would have been impossible to chase the label from D-glucitol, if the fermentation of the yeast had been the source of the labeled D-glucitol.

The configuration of D-glucose in ADPGlc is α and is retained when the glucose is incorporated into a starch chain. To obtain this, it is postulated that D-glucose forms a labile β linkage with the starch synthase. The pulse and chase studies and the study of the pH 2 treatment, indicates that starch chains are biosynthesized by the D-glucose and the starch chain both forming high-energy, covalent complexes with the enzyme, starch synthase. The pulse and chase data further show that D-glucose from ADPGlc is added to the reducing end of the growing starch chain, increasing its size by a D-glucose residue.

To obtain the synthesis of a starch chain with the above-mentioned conditions, it is proposed that the elongation process occurs by a two-site, insertion mechanism in which D-glucose is transferred from ADPGlc to two sites at the active-site of the enzyme, forming covalent linkages with the enzyme (Fig. 2). The C-4 hydroxyl group of one of the glucose units then makes a nucleophilic attack onto C-1 of the other glucose unit, forming an α-(1→4) linkage between the two glucose units and the formation of a maltosyl unit attached to the enzyme. The free site on the enzyme then attacks another ADPGlc to give a new glucopyranosyl–enzyme complex, whose C-4 hydroxyl then attacks C-1 of the maltosyl unit to give another α-(1→4) glycosidic linkage and the formation of a maltotriosyl unit attached to the enzyme. These reactions then continue, going back-and-forth between the two sites, to give the formation of a starch chain, until the chain is removed from the enzyme active-site by hydrolysis (see Fig. 2).

The pulse and chase data clearly show that the primer-dependent mechanism, in which D-glucose would be added to the nonreducing end of a primer chain, is not involved in the synthesis of starch, as has been assumed, but never demonstrated, for over 60 years. If the primer-dependent mechanism were involved, it would be impossible to obtain any labeled D-glucitol in the pulse reaction, as all of the D-glucose residues would be added to the nonreducing ends of the

elongated primer chains (see Fig. 1(A)). Likewise, the alternative primer mechanism in which it is postulated that D-glucose is added to a protein primer to give an oligosaccharide chain of 6–10 D-glucose residues, which then acts as a classical primer and D-glucose residues from ADPGlc are added to the nonreducing ends of the oligosaccharide primer (see Fig. 1(C)), is also not taking place. If this were the mechanism of chain elongation, the reducing end D-glucose residue could become labeled in the pulse reaction and give labeled D-glucitol, but the amount of label in D-glucitol would remain constant, at a relatively low value, throughout the reaction and could not be decreased by a chase reaction, over time, because the nonlabeled chase D-glucose would also be added to the nonreducing end of the primer and not to the reducing end of the chain.

The enzymes present in the starch granules most probably were incorporated in the granules when the granules were first being formed in the plant, and they most probably represent the enzymes that were involved in the formation of the granules. The enzymes remain active over long periods of time, apparently stabilized by the products of their action, the starch chains. The presence of active starch synthesizing enzymes in starch granules has provided a convenient model system for studying the mechanism of starch biosynthesis. The granules are easily handled; they can be weighed and the enzyme activity assayed; they can be removed from aqueous suspension in buffer by centrifugation and washed free of soluble materials; radioactivity that has been incorporated during pulse and chase reactions can be easily determined by heterogeneous liquid scintillation spectrometry; the granules are readily permeable to any low molecular weight constituents, such as acids, bases, buffers, and substrates; and the granules can be readily solubilized by heating in water.

The addition of activated D-glucose from ADPGlc to the reducing end of a growing starch chain, via the insertion mechanism, appears universal for starch biosynthesis in that labeled D-glucitol was obtained from the pulse reactions and was decreased in the chase reactions for all of the eight diverse varieties of starch granules that were studied. These different varieties of starch have different physical and chemical properties and different kinds and amounts of starch synthase isoforms. Starch now joins the growing number of polysaccharides (*Salmonella* O-antigen polysaccharide, bacterial cell-wall murein, dextran, alternating comb dextran, mutan, bacterial cellulose, and xanthan) that have been shown to have their polysaccharide chains elongated from the reducing end by a two-site insertion mechanism.

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