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Tests for the mechanism of starch biosynthesis: de novo synthesis or an amylogenin primer synthesis

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ABSTRACT

Studies in 1940 on potato phosphorylase reaction with starch found that D-glucopyranose from α-D-glucopyranosyl-1-phosphate was added to the nonreducing-ends of starch chains. This led to the hypothesis that the biosynthesis of starch required a preformed primer. Later it was found that phosphorylase was exclusively a degradative enzyme in vivo and that starch-synthase was the enzyme that reacted with ADPGlc to biosynthesize starch. Amylogenin, a putative self-glycosylated protein, was postulated to be the primer, although it was never demonstrated or found. In the present study, three reactions were performed in sequence with a highly purified potato starch-synthase to determine whether an amylogenin primer was present and required or whether the biosynthesis was de novo. Reaction 1 was performed by adding 2.0 mM ADPGlc to synthesize the putative primer to a possible amylogenin in the preparation; in Reaction 2, 10 mM ADP-[^{14}C]Glc was added; and in Reaction 3, 10 mM nonlabeled ADPGlc was added. After the isolation, reduction, and acid hydrolysis of the products of Reactions 2 and 3, ^{14}C-D-glucitol was obtained from Reaction 2 and was decreased by Reaction 3. The formation of ^{14}C-D-glucitol and its decrease showed that an amylogenin, protein primer was not involved in starch biosynthesis and the synthesis is de novo by the addition of D-glucose to the reducing-ends of growing starch chains.

1. Introduction

In 1939, Cori and Cori[1] reported that glycogen muscle phosphorylase catalyzed the reaction of inorganic phosphate with glycogen to give α-D-glucopyranosyl-1-phosphate (α-D-Glc-1-P). Almost simultaneously in 1940, Hanes[2] was also studying potato phosphorylase and the inorganic phosphate reaction with starch to give α-D-Glc-1-P. At this time, Hanes[2] also observed that if he started with phosphorylase and α-D-Glc-1-P and starch, α-D-glucopyranose was added to the nonreducing-ends of the starch chains. Hanes further found that when he started with only α-D-Glc-1-P and phosphorylase, no reaction was obtained. In 1947, Swanson and Cori[3] also reported that muscle phosphorylase catalyzed the addition of α-D-glucopyranose from α-D-Glc-1-P to the nonreducing-ends of glycogen chains and without glycogen no synthesis was obtained. These potato and muscle phosphorylase reactions, starting with α-D-Glc-1-P and either starch or glycogen, were considered to be the mechanism for the biosynthesis of starch and glycogen, respectively, and preformed starch, glycogen, or maltodextrins were required primers for the syntheses.

For a time, it was thought that phosphorylase was the synthetic enzyme for both starch and glycogen biosynthesis. It, however, was found that when potato or glycogen phosphorylase was conducted in the synthetic direction, that is, starting with α-D-Glc-1-P and preformed starch or glycogen, the reaction rapidly slowed down, as the concentration of P_i increased, and stopped after the addition of only three or four D-glucopyranosyl units to the nonreducing-ends of the starch and glycogen chains. It was then shown that the equilibrium ratio[4] of P_i to α-D-Glc-1-P at pH 7.0 was 3.6 and the concentration of inorganic phosphate in plants and animals was 20- to 40-fold higher than α-D-Glc-1-P.[5] This indicated that neither starch nor glycogen was biosynthesized in vivo by potato phosphorylase or muscle phosphorylase, respectively, and the reaction was exclusively degradative rather than synthetic. Nevertheless, the concept of a primer for polysaccharide biosynthesis was established in the minds of many polysaccharide investigators and the primers were futilely and unsuccessively sought for many years.

In 1957, Leloir and Cardini[6] discovered that an enzyme, glycogen-synthase, reacted with UDPGlc and catalyzed the biosynthesis of glycogen; and in 1961, Recondo and Leloir[7] found that starch-synthase catalyzed the biosynthesis of starch by reaction with ADPGlc. This led to a search for the glycogen and starch primers that were assumed to be required for their biosynthesis.

It, however, was not until 1975 that Krisman, and Bargengo[8] reported that rat heart tissue produced a protein with covalently linked α-1,4-glucan that was produced by reaction of the protein with UDP-[^{14}C]Glc. It was then proposed that this protein–carbohydrate product was the long sought primer for the

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biosynthesis of glycogen. Observations similar to Krisman and Barengo[8] were also reported for *Escherichia coli*,[9] *Neurospora crassa*,[10] and rabbit muscle and rabbit liver,[11] and *Saccharomyces cerevisiae* yeast.[12]

Evidence was obtained that the protein self-glucosylated itself, giving a maltodextrin having 7–11 D-glucose units that acted as the primer for glycogen biosynthesis. The glycosylated protein was eventually named, glycogenin.[11]

In 1975, Cardini and co-workers[13,14] also found that a potato particulate preparation from proplastids was glycosylated by UDP-[^{14}C]Glc to give a protein that had short ^{14}C-labeled glucan chains. It, however, was never shown that this was the protein primer for starch biosynthesis. In 1994, Rothschild and Tandecarz[15] obtained a 38 kDa protein from maize endosperm that was glucosylated by UDP-[^{14}C]Glc and it was proposed to be the primer for maize starch biosynthesis. This glucosylated protein was named amylogenin,[14] but it also turned out to be a reversibly glucosylated protein located in the Golgi that was involved in the biosynthesis of the cell wall matrix, rather than in the biosynthesis of starch.[15–17] In 2009, Whelan stated that 'to the best of our knowledge, the primer for starch synthesis has still not been found'.[18]

In 2010, Robyt and co-workers[19] reported several purified starch-synthase fractions from potato tubers. These purified starch-synthase fractions were shown to be free of any carbohydrate putative primers and synthesized starch chains de novo, without the addition of any carbohydrate primers.[20] This study further showed that both D-glucopyranosyl and glucanosyl-chains were formed and were covalently linked to the active-site of starch-synthase; and during reaction, the D-glucopyranosyl units were added by a transglycosylation reaction, to the reducing-ends of the growing, covalently linked glucanosyl-chains. This polymerization reaction was shown to be processive. A two catalytic-site insertion mechanism was proposed to explain the mechanism for the biosynthesis of starch, showing the *initiation,* the *polymerization,* and the *termination* reactions.[20]

This study, however, produced a dichotomy, as to why 61-studies involving starch biosynthesis from 1964 to 2012, have had to add a carbohydrate primer;[21] glycogen was the best primer and maltodextrins (maltotriose–maltoheptaose) were not as good primers.[22] Glycogen, however, cannot give starch. Mukerjea and Robyt[20] showed that potato starch-synthase was free of any carbohydrate primer and synthesized starch chains de novo without the addition of any primers. It was found that all of the 61-studies had used 25−100 mM Tris, Bicine, or Tricine buffers.[21] Robyt and co-workers[21] then showed that the Tris-buffers completely inhibited starch-synthase at the concentrations used. It was also shown that the addition of 10 mg/mL of glycogen or maltotetraose putative primers gave only a partial reversal (~10–15%) of the inhibition and that the Tris-type buffers formed a complex with the ADPGlc substrate, removing it from the enzyme digests, and causing the inhibition. The addition of the putative primers or 500 mM Na-citrate[23] partially released some of the ADPGlc substrate from the Tris-type buffer-ADPGlc complexes, permitting it to act as a substrate, giving partial activity from that of a controlled amount of purified starch-synthase, without Tris-buffers.[21] There still remained, however, the question as to whether a protein–carbohydrate primer, amylogenin, similar to glycogenin, was required for starch biosynthesis.

In the present study, we investigated two possible mechanisms for starch biosynthesis: (1) the formation of a glucosylated amylogenin primer and the addition of D-glucose to the nonreducing-ends of the primer, and (2) the de novo synthesis of starch by starch-synthase without the requirement of a primer and the addition of D-glucose to the reducing-end of a growing starch chain. Three experiments were performed in sequence, with purified potato starch-synthases,[19] 250 mIU and 500 mIU of T1F23 and 500 mIU of T2F19 from Ref. 19. The following three reactions were performed with the starch-synthase fractions: Reaction 1, a reaction with 2 mM ADPGlc was performed to synthesize the putative maltodextrin-primer, attached to the putative amylogenin-protein; Reaction 2, a reaction with 10 mM ADP-[^{14}C]Glc; and Reaction 3, a chase reaction with 10 mM nonlabeled ADPGlc.

2. Experimental

2.1. Materials

Dithiothreitol (DTT); polyvinyl alcohol 50 K (PVA); and PPO and PoPoP were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Adenosine 5′-diphospho-α-D-glucopyranose (ADPGlc) was obtained from Carbosynth LLC (19535 West Bernado Drive, Suite 226, San Diego, CA 92127, USA). ADP-[^{14}C]Glc (333 mCi/mmol) was obtained from American Radiolabeled Chemical, Inc. (101 Arc Drive, St. Louis, MO 63147, USA). Liquid scintillation cocktail was prepared, containing 5.0 g of PPO and 0.1 g of PoPoP in 1 L of toluene. All chemicals were of the highest grade.

Two starch-synthase fractions, 23 and 19 (Table 1, Ref. 19), and substrates and buffer were shown to be free of any carbohydrate primer[20] using the Whelan technique of treatment with immobilized α-amylase and immobilized glucoamylase.[24] Standard buffer (pH 8.5), contained 10 mM glycine, 2.0 mM EDTA, 1.0 mM DTT, and 0.04% (w/v) polyvinyl alcohol 50 k.[19]

2.2. Methods

2.2.1. Assay of starch-synthase

The assay was performed in 75 μL of standard buffer (pH 8.5), containing 20 mM (0.05 μCi) ADP-[^{14}C]Glc; to which 25 μL of starch-synthase was added and incubated at 37 °C for 30 min; 25 μL of aliquots were removed and 2.5 μL of 1.0 M NaOH was added to stop the reaction. This was then added to 1.5 cm-square Whatman 3 MM paper, which was immediately added to 100 mL of MeOH, with stirring for 10 min, to precipitate the synthesized amylose onto the paper and remove soluble materials, such as unreacted ADPGlc, ADP, and buffer components. The paper was washed two more times with 100 mL of MeOH, dried, and counted in toluene cocktail. Background controls were obtained by adding 2.5 μL of 1 M NaOH to 25 μL of the standard-Buffer solution, containing the ADP-[^{14}C]Glc substrate, without the enzyme, followed by adding it to a Whatman 3MM 1.5 cm paper square.[19,25] From the hundreds of assays for starch-synthase that we have performed, using ^{14}C-radioisotope, ADP-[^{14}C]Glc, it has been determined that the precision of the measurements is 1–2 parts/1000. 1.0 mIU of starch-synthase = 1.0 nmol of D-glucose from ADPGlc is incorporated into starch per min.

2.2.2. Experiments to test for an amylogenin primer mechanism or reducing-end de novo biosynthesis mechanism by starch-synthase

2.2.2.1. Reaction 1 of 2.0 mM ADPGlc to form a putative amylogenin protein primer. A solution of 1.2 mg of ADPGlc was dissolved in 150 μL of Standard Buffer and preincubated at 37 °C; and 850 μL of fraction 23 (Table 1, Ref. 19) is also preincubated at 37 °C. The reaction was initiated by adding the ADPGlc (150 μL) to the enzyme fraction (850 μL), giving 250 mIU or 500 mIU of starch-synthase and 2.0 mM ADPGlc in 1000 μL. The reaction was run at 37 °C for 24 min (1.0 CP), where 1.0 CP is the conversion period (time) to theoretically convert all of the reactants into products.

2.2.2.2. Reaction 2, with 10 mM ADP-[^{14}C]Glc. A solution with 7.1 mg (0.25 μCi) ADP-[^{14}C]Glc in 200 μL of Standard Buffer