OPEN QUESTIONS AND HYPOTHESES IN STARCH BIOSYNTHESIS

What roles do the starch synthase isoforms play in the formation of the crystalline starch granule and amylopectin structures? How is amylose formed? Why are starch granules from different species different in size and in the number per cell? New methodology and much effort have resulted in major advances in the understanding of starch biosynthesis, but many questions remain unanswered. Here we discuss some of these open questions and possible answers.

I. INITIATION OF STARCH BIOSYNTHESIS

The synthesis of starch as described in the chapter, "The Biosynthetic Reactions of Starch Synthesis," requires the presence of a glucan primer, that is, a glucan chain that is then elongated by the addition of a glucosyl group in the following reaction:

$$\text{ADP-glucose} + [\text{glucosyl}]_n \rightarrow \text{ADP} + [\text{glucosyl}]_{n+1}$$

This equation, as written, poses the question of how the primer glucosyl$_n$ is first formed. It seems, however, that many enzymes capable of elongating glucans can display activity independent of added primer when assayed under suitable conditions, especially in the presence of citrate. Whether these unprimed reactions proceed in vivo is unknown. Citrate has been shown to decrease, by a large factor, the K_m for primer of several enzymes: the chloroplastic starch phosphorylase (Sivak, 1992), and some starch (Boyer and Preiss, 1979; Pollock and Preiss, 1980) and glycogen (Fox *et al.,* 1976; Holmes and Preiss, 1979) synthases. Because enzyme preparations often contain some glucan brought from the material from which they were isolated, activity independent of added primer is unlikely to represent de novo synthesis of carbohydrates in most of the systems examined. In the case of the glycogen synthase from *Escherichia coli,* which was studied in great detail, the glucose is incorporated into minute amounts of glucan primer that is associated (Fox *et al.,* 1976), but is not covalently bound

(Holmes and Preiss, 1979) with the enzyme. A similar situation is that of the chloroplastic phosphorylase of spinach leaf (Sivak, 1992).

Common criteria for the identification of an unprimed product as a proteoglucan have been tested and are discussed elsewhere (see, e.g., Sivak, 1992)—that is, precipitation with trichloroacetic acid (TCA), hydrolysis with dilute 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. A property of many amyloses, and of starches in general, can lead to confusion and artifacts. During electrophoresis, a small part of the polysaccharide migrates into the polyacrylamide gel in the presence of the sodium dodecyl sulfate and the urea used to break noncovalent bonds. The presence of the polysaccharide can be revealed by using an iodine reagent, with the color depending on the degree of ramification of the polyglucose chain. This peculiar property of polysaccharides, which mimics protein behavior, has been attributed to the capacity of these glucans to form complexes with the negatively charged sodium dodecyl sulfate (SDS) (as they do with iodine). Also, the products of phosphorylase and of glycogen and starch synthase independent of added primer are long, mostly linear α-1,4-glucans, which because of their linearity, tend to precipitate in aqueous 5% or 10% TCA, a property that can mislead researchers into believing them to be glucosylated proteins.

Is the reaction independent of added primer relevant? This question cannot be answered, but mention can be made of some matters that must be resolved before it can be answered. The first is whether in vivo glycogen and starch synthases are able to use ADPGlc in the absence of glucan (i.e., whether the conditions are favorable for the reaction). The second is whether the site of starch synthesis, the amyloplast, is ever completely devoid of glucans that could act as primers for the starch synthase.

Speculation notwithstanding, enzymes have been isolated from potato tuber and maize endosperm, which catalyze the formation of a primer suitable for starch synthases. Studies using a particulate (sedimentable at high speed, membrane-containing) fraction of potato tuber provided evidence for the synthesis of α-1,4-glucosidic chains covalently bound to protein. The glucosyl donors in these reactions were UDPglucose, ADPglucose, and glucose-1-P (Lavintman and Cardini, 1973; Lavintman *et al.*, 1974). On the basis of other studies (Tandecarz and Cardini, 1978, 1979), it was proposed that, at least in potato tuber, a two-step reaction occurred involving a protein glucosyl acceptor, as follows:

1. UDPglucose + acceptor protein $\rightarrow$ acceptor protein-glucose + UDP
2. acceptor protein-glucose + nADP(UDP)glucose/glucose 1-P $\rightarrow$ acceptor protein-glucose-(glc)n + nADP(UDP) or nP_i

The first reaction would be catalyzed by a UDPglucose : protein transglucosylase (UPTG), and the second reaction by either a starch synthase or a phosphorylase. Moreno *et al.* (1986) solubilized and partially purified the components that catalyzed reaction 1. Ardila and Tandecarz (1992) and Bocca *et al.* (1997) purified UPTG to electrophoretic homogeneity. It is not certain whether the transglycosylase and the acceptor protein are different or the same molecule. If they are one, then the transglucosylase must self-glucosylate, as suggested by the fact that an apparently pure fraction conserved catalytic activity. The acceptor protein was determined to have a molecular mass of 38,000, and only one glucose moiety was transferred to the protein. A β-elimination reaction carried out in the presence of a reducing agent showed that an *O*-glucosidic linkage was formed and that the amino acids Ser and Thr were involved; the reaction required $MnCl_2$. Specific phosphorylases and starch synthases in the potato tuber were able to use the product of reaction 1, the glucosylated acceptor protein, as a primer to synthesize α-1,4-glucan chains (Moreno *et al.*, 1987).

The reactions involving the UDPglucose : protein transglucosylase are similar to the reactions proposed for the initiation of glycogen synthesis in mammals (Pitcher *et al.*, 1987, 1988; Lomako *et al.*, 1988). Glycogenin, a 37-kDa protein, in association with glycogen synthase, self-glucosylates to form a glucosyl protein that can act as a primer for the glycogen synthase. The glycogenin protein has been sequenced (Campbell and Cohen, 1989) and, in contrast to the plant acceptor protein, the glucosidic linkage formed is with a tyrosine hydroxyl group (Smyth, 1988). Krisman (1972) was the first to postulate that the de novo synthesis of glycogen required an initiating protein factor.

The results obtained by Cardini, Tandecarz, and their collaborators in the plant system are exciting, but some important questions remain. As we know, starch synthesis occurs in plastids (i.e., chloroplast or amyloplast). Where in the cell is the transglucosylase/acceptor protein located? If it is in the plastid, then the glucosyl donor, UDPglucose, is not available for glucosylation because it is made in the cytosol and does not enter readily the chloroplast or amyloplast. It is possible, however, that the glucosylation may occur in the cytosol and then the glucosylated acceptor protein translocates into the plastid. It is also important to note that thus far these reactions have only been demonstrated in potato tuber and maize endosperm and not in other species or organs. When cDNA coding for this protein is cloned (antibodies against the protein have been raised; J. Tandecarz, personal communication), antisense experiments will be possible. Antisense RNA is a transcript that has a high degree of complementation with a target mRNA so that it can hybridize with the mRNA in vivo. In this way, the antisense RNA acts as a repressor of the function of the target RNA. These experiments, however, are difficult to interpret because it is virtually impossible to obtain zero expression and because often plant metabolism

accommodates by using an alternative pathway. If the 38-kDa protein associated with UPTG is also present in *Chlamydomonas reinhardtii* then the next step would be to find mutants lacking the 38-kDa protein and see whether starch is still synthesized in the mutant. The use of gene disruption in Antirrhynum nafus is also an attractive possibility (see Experimental Systems in the study of starch metabolism).

II. HOW IS THE STARCH GRANULE FORMED?

The glycogen-synthesizing enzymes from *E. coli* are not much different from those in plants, but bacteria make glycogen and not starch. When the maize-branching enzymes are expressed in *E. coli,* they conserve the properties they have in the plant, but the resulting glucan is glycogen; not starch. How is the intrincate structure of the starch granule formed in vivo?

The sugary mutants of maize have been known since the beginning of the twentieth century. The mutant accumulates about 35% of its dry weight as phytoglycogen, a highly branched, water-soluble polysaccharide. Phytoglycogen has 7 to 10% of its glucosidic linkages as α-1 $\rightarrow$ 6, and is therefore more highly branched than amylopectin (Manners, 1985). Pan and Nelson (1984) found that all the *su* 1 mutants were deficient in a particular endosperm-debranching enzyme activity, pullulanase, suggesting that the debranching activity is the biochemical deficiency leading to phytoglycogen formation. The debranching enzymes of normal maize endosperm were separated into three peaks of activity on a hydroxyapatite column and it was found that the *su* 1 mutant lacked one of the activity peaks toward pullulan, whereas the other two peaks were also much reduced in activity. The debranching enzyme activity of developing endosperms is proportional to the number of copies of the *Su* 1 gene, suggesting that the *Su* 1 gene is the structural gene for the debranching enzyme. The debranching enzymes, however, have not been characterized to a great extent (but see Lee *et al.*, 1971).

The observation that debranching enzyme deficiency was associated with the presence of phytoglycogen in the *su* 1 mutants (Pan and Nelson, 1984) revived a hypothesis of Erlander (1958), who proposed that amylopectin synthesis was due to debranching of phytoglycogen, which was first formed via starch synthase and branching enzyme catalysis. Although phytoglycogen may not be a normal intermediate in the synthesis of amylopectin, this may occur from a more highly branched α-1 $\rightarrow$ 4-glucan that is formed via the action of the starch synthase and branching enzyme isoforms. If the activity of the debranching enzyme is insufficient, then a more highly branched, water-soluble glucan could accumulate with a concomitant de-

crease in the amylopectin component of the starch granule and, possibly, an increase in the relative amylose content of the granule.

The hypothesis that the *sugary* 1 mutation affects the structural gene for a debranching enzyme is further supported by the isolation of a cDNA of the *su* 1 gene. However, its deduced amino-acid sequence is similar to a bacterial isoamylase (James *et al.,* 1995) rather than to a pullulanase.

In *C. reinhardtii,* Ball and his collaborators (Mouille *et al.,* 1996) generated seven independent alleles in the *sta*7 locus. All mutants lacked granular starch, but contained a water-soluble polysaccharide, similar to maize phytoglycogen, in an amount equivalent to 5% of the starch content of the wild type. This defect was associated with the disappearance of a specific debranching activity. All other starch-related enzyme activities were normal.

It remains to be shown whether the *su* 1 gene product debranching enzyme activity is actually an isoamylase or a pullulanase (Hizukuri, 1995). Isoamylases, such as that present in *Bacillus amyloderamosa,* readily debranch amylopectin. Pullulanase, such as that present in *Aerobacter aerogenes,* completely debranches amylopectin, but its action on glycogen is usually incomplete. The specificity of these reactions should be studied further with respect to the factors that determine which α-1,6 linkages are cleaved and which remain resistant to debranching action. It is possible that the crowding of the α-1,6 linkages in a cluster region in amylopectin causes some steric difficulties for the debranching of the linkages in the cluster region, but at present this is only conjecture. As for the problem of starch initiation, this is a research field that would benefit greatly from gene disruption experiments.

III. A COMPLETE PATHWAY

From the information available on the branching enzyme and starch synthase isozymes, a possible route for the synthesis of amylopectin and amylose can be proposed as shown in Fig. 1.

A reaction with the potential of being the initiating reaction for synthesis has been observed in potato tuber and maize endosperm (see preceding). The resulting glucosylated 38-kDa protein can serve as a primer for the synthesis of starch via the starch synthase reactions. Whether there is an acceptor protein that could be glucosylated by ADPGlc has not been demonstrated; the proposed initiating reaction and acceptor protein have not been characterized as well as the other reactions in starch synthesis.

After the formation of the unbranched maltosaccharide–protein primer of undetermined size, high rates of polysaccharide formation may occur at

1. Initiation of synthesis of unbranched maltodextrins (bound to protein?)

2. Unbranched maltodextrin + ADPGlc ---(GBSS + SSSII + BEI)---> Long, intermediate size chains of glucan

3. Long, intermediate size chain glucan + ADPGlc ---(BEII + SSS I)---> Synthesis of A & shorter B Chains to finish cluster Structure

4. Repeat of reactions 2 and 3 to form the complete pre-amylopectin polysaccharide.

5. pre-amylopectin structure ---(Debranching Enzyme)---> amylopectin + pre-amylose chains

6. pre-amylose chains ---(GBSS)---> amylose

FIG. 1. Hypothetical pathway for the synthesis of amylose and amylopectin. Initiation may involve synthesis of a maltodextrin attached covalently to a protein. This putative protein-α-glucan then can accept glucose from ADPGlc, either via GBSS catalysis to form an amylose structure, or in combination with BEI, SSSII, and (possibly) GBSS to form a polysaccharide having the internal structure of the final amylopectin product. BEII and SSSI carry out the reactions to form the exterior of the amylopectin structure. The enlargement of the amylopectin could proceed further by continuing participation of BEI, SSSII, and (possibly) GBSS, by repeat of reactions 2 and 3. Production of amylopectin in reaction 5 is caused by debranching enzyme, which also generates oligosaccharide chains, which are elongated by GBSS to form the amylose fraction.

the surface of the developing starch granule, where granule-bound starch synthase (GBSS), soluble starch synthase II (SSSII), and branching enzyme I (BEI) interact with the glucosylated protein primer to form a branched α-glucan containing both long and intermediate-size chains.

The postulation of phase 2 in Fig. 1 is based on the studies of the polysaccharide structures observed in the *C. reinhardtii* mutants deficient in SSSII and GBSS (Fontaine *et al.*, 1993; Maddelein *et al.*, 1994a,b), as well as the *ae* mutants of rice (Mizuno *et al.*, 1993) and maize (Boyer and Preiss, 1981), which are defective in branching enzyme II (BEII). BEII deficient mutants have altered polysaccharides with fewer branches and longer sized branched chains. In phase 3, SSSI and BEII are responsible for the synthesis of the A- and exterior B-chains to complete the first cluster region in the glucan. Continued synthesis in phase 4 is essentially a repeat of phases 2 and 3 to synthesize a highly branched α-glucan, termed *proamylopectin.* This highly α-branched glucan is water soluble and noncrystalline. In phase 5, a debranching enzyme debranches the preamylopectin to form amylopectin, which can now crystallize. In phase 6, the chains, liberated by debranching action of the proamylopectin could be used as primers by GBSS to form amylose. Amylose synthesis may occur only inside the starch

granule, and only GBSS would be involved because it may be the only starch synthase present at the site of amylose synthesis.

These reactions do not have to occur in perfect sequence, and the phases may have some overlap (e.g., phases 2, 3, and 4 may overlap, and possibly even 5 and 6). However, the present evidence, such as intermediate products formed by starch mutants of *C. reinhardtii* and of higher plants, supports the sequence of reactions shown in Fig. 1 for amylopectin and amylose biosynthesis. Further experiments are required to test this hypothetic scheme, and attempts to purify and characterize the debranching enzyme, crucial to this hypothesis, are under way.

It should be noted that the proamylopectin in this still hypothetical pathway would be larger than the phytoglycogen found in the mutants lacking debranching activity. This is because proamylopectin would have a size comparable to amylopectin, while phytoglycogen, much smaller, may be the product of degradation of a proamylopectin unable to crystallize into amylopectin and may be so unprotected that it would be subject to the action of amylases.