BIOSYNTHETIC REACTIONS OF STARCH SYNTHESIS

I. INTRODUCTION

The metabolic routes leading to polyglucan synthesis were elucidated after the discovery of nucleoside-diphosphate sugars by L. F. Leloir and co-workers in 1955. This finding led to the conclusion that biosynthesis and degradation of glycogen and starch occur by different pathways.

In mammalian cells, glycogen synthesis is relatively well understood; glycogen synthase is specific for UDPglucose and is regulated through hormonally induced posttranslational protein modification. Textbooks of biochemistry usually describe these metabolite schemes in detail. The biosynthesis of polysaccharides in bacteria and plants is, in contrast, usually described in less detail. These organisms accumulate glycogen (bacteria) or starch (plants) by metabolic pathways that are different than those occurring in animals. Despite the difference in the final product (glycogen or starch), in bacteria and in plants ADPglucose is the glucosyl donor for the elongation of the α-1,4-glucosidic chain. Moreover, in both plants and bacteria, the main regulatory step of the metabolism takes place at the level of ADPglucose synthesis.

II. PIONEERING STUDIES

The formation of α-1,4-glucosidic linkages in vitro by plant enzymes was first demonstrated in 1940, when Hanes showed that potato tuber extracts formed an amylose-like product with glucose-1-P as a glucosyl donor (reaction 1).

$$\text{glucose-1-P} + \alpha\text{-glucan primer} \Leftrightarrow \text{Pi} + (1\rightarrow4)\text{-}\alpha\text{-glucosyl-glucan} \quad (1)$$

Since then, phosphorylase activity has been found to be ubiquitous in plant extracts, but its role in vivo is now believed to be starch degradation rather than synthesis.

In the 1960s, L. F. Leloir, C. E. Cardini, and their collaborators in Buenos Aires, Argentina, demonstrated the synthesis of α-1,4-glucosidic linkages by

a plant extract using either UDPglucose (Leloir *et al.*, 1961) or ADPglucose (Recondo and Leloir, 1961; Frydman and Cardini, 1967) as glucosyl donors (reaction 2), which is catalyzed by the starch synthase (EC 2.4.1.21).

$$\text{ADP(UDP)glucose} + \alpha\text{-glucan} \rightarrow \text{ADP(UDP)} + (1\rightarrow4)\text{-}\alpha\text{-glucosyl-glucan} \quad (2)$$

Since then, starch synthase activity has been reported to be present in many plant extracts (for reviews, see Preiss and Levi, 1980; Preiss and Sivak, 1996).

The sugar nucleotides UDPglucose and ADPglucose can be synthesized in plants either by a pyrophosphorylase-type reaction (reactions 3a and 3b; Espada, 1962) or via a reversal of the sucrose synthase reaction (reaction 4; Cardini *et al.*, 1955; de Fekete and Cardini, 1964)

$$\alpha\text{-glucose-1-P} + \text{ATP} \Longleftrightarrow \text{ADPGlc} + \text{PP}_i \quad (3a)$$

$$\alpha\text{-glucose-1-P} + \text{UTP} \Longleftrightarrow \text{UDPGlc} + \text{PP}_i \quad (3b)$$

$$\text{sucrose} + \text{ADP(UDP)} \Longleftrightarrow \text{fructose} + \text{ADP(UDP)glucose} \quad (4)$$

Formation of the α-(1→6) linkage branch points present in amylopectin and phytoglycogen is catalyzed by the branching enzyme (EC 2.4.1.18; Bourne and Peat, 1945; Hobson *et al.*, 1950), also called the Q enzyme.

$$\text{linear glucosyl chain of } \alpha\text{-glucan} \rightarrow \text{branched chain of } \alpha\text{-glucan with } \alpha\text{-1}\rightarrow\text{6-linkage branch points} \quad (5)$$

III. THE ADPglucose PATHWAY IS THE MAJOR PATHWAY OF STARCH SYNTHESIS *in Vivo*

Which of the enzymatic activities mentioned previously are involved in starch synthesis *in vivo?* To accept that an enzyme is a likely component of the pathway in the plant itself, it must fulfill the following criteria:

1. On careful extraction (i.e., avoiding proteolysis, inactivation by phenolics), from the plant tissue, the maximal activities measured *in vitro* (i.e., in the presence of activators at optimum pH) should at least equal the rates of starch synthesis measured *in vivo.*
2. The enzyme should be in the right compartment within the cell.
3. The kinetic characteristics (i.e., affinity for the substrate, effect of activators and inhibitors, pH optimum of the enzyme should be compared

with the concentrations of these substrates and modifiers in the site of starch synthesis (i.e., the chloroplast or amyloplast). This comparison will help in determining whether the activity in situ is likely to be sufficient to support the actual rate of synthesis measured in vivo. Calculation of the in vivo concentration of a particular metabolite is not an easy task; it involves the isolation of the organelle in question with minimum disruption and the avoidance of postisolation changes. With the nonaqueous techniques, the tissue is quickly frozen and the composition of the different components is assumed to be unchanged throughout the nonaqueous fractionation. The aqueous methods rely on fast separation of the different compartments with minimal cross-contamination.

4. Mutations resulting in the loss of a relevant enzyme should result in a commensurate decrease in starch content or a significant change in starch structure.

5. There should be a correlation between increases in the relevant enzymatic activities and the accumulation of starch during the development of the tissue (e.g., the potato tuber or the maize seed).

With these statements in mind, it is easier to address the reports proposing that UDPglucose-specific starch synthases and starch phosphorylases may be involved in starch synthesis. Their high K_m values for their substrates (UDPglucose and glucose-1-P, respectively), as compared to concentration in the relevant cellular compartments, argues against a significant role in starch biosynthesis. In addition, the synthesis of UDPglucose, at least in the starch-synthesizing plant tissues studied so far, occurs in the cytosol and not in the amyloplast, and no significant transport of UDPglucose into the plastid has been reported. Phosphorylase catalyzes an equilibrium reaction in cells that have P_i concentrations in excess of glucose-1-P, indicating that it plays a role in starch degradation rather than in synthesis.

Data from a number of genetic and biochemical studies indicate that the ADPglucose pathway, involving the reactions described in the preceding text is very important for starch synthesis. Mutants of maize endosperm *shrunken* 2 and *brittle* 2 (Tsai and Nelson, 1966; Dickinson and Preiss, 1969b), which are deficient in ADPglucose pyrophosphorylase (ADPGlc PPase) activity, are also deficient in starch. Smith *et al.* (1989) have shown that a pea line having recessive *rb* genes (genes controlling the level of ADPGlc PPase activity in developing pea embryos), containing 3–5% of the ADPGlc PPase activity, had only 38 to 72% of the starch found in the normal pea line. In *Arabidopsis thaliana,* Lin *et al.* (1988b) isolated a mutant containing less than 2% of the starch seen in the normal strain and less than 2% of the ADPGlc PPase activity. Immunoblots indicated that the enzyme was absent from the *Arabidopsis* extracts. In the potato tuber,

Müller-Röber and colleagues (1992) expressed a chimeric gene encoding antisense RNA for the ADPGlc PPase small subunit, which caused a reduction in enzymatic activity of 2 to 5% of the normal levels, which led to a reduction in starch content.

Thus, in four different plant systems, a reduction of ADPGlc PPase activity led to a reduction in starch accumulation. Alternatively, an increase in ADPGlc PPase activity was achieved by transformation of the potato tuber with a mutant *E. coli* ADPGlc PPase gene that was insensitive to the regulatory effectors of the plant enzyme (Stark *et al.*, 1992). This increased the potato tuber starch content by 30 to 60%, suggesting not only that the role of the ADPGlc PPase in starch synthesis is important, but also that the enzyme activity is normally rate limiting. The introduction of the bacterial gene into tomato fruit (Stark *et al.*, 1992) and into safflower seed (G. Kishore, personal communication, 1997) also increased their starch content dramatically. Other data showing a relationship between activity of the ADPGlc PPase and starch accumulation in other plant species have been previously reviewed (Preiss and Levi, 1980; Preiss, 1988, 1991; Okita, 1992; Sivak and Preiss, 1995; Preiss and Sivak, 1996). Thus, the ADPGlc PPase and the subsequent reactions utilizing ADPglucose are the dominant routes for starch synthesis in plants, and ADPglucose synthesis is perhaps rate limiting. In the case of starch phosphorylase, the first criterion is fulfilled, but the concentrations of P_i and glucose-1-P in the amyloplast and chloroplast are considered to be more compatible with a role of the enzyme in degradation rather than in synthesis. No correlation between plastid phosphorylase activity and starch accumulation has been found. No mutants deficient in starch synthesis have been found that are deficient in phosphorylase. Some reports suggest that phosphorylase may play some role in starch synthesis (Obata-Sasamoto and Suzuki, 1979; Mengel and Judel, 1981), a conclusion based on the fact that phosphorylase levels were higher than starch synthase and/or ADPGlc PPase. It should be noted, however, that insensitive assays for the ADPglucose enzymes were frequently used, and that although phosphorylase was found to be higher in activity, the physiologic concentrations of P_i and glucose-1-P make it unlikely to ever function in a synthetic pathway. The equilibrium constant for phosphorylase is 2.4 at pH 7.3 (Cohn, 1961). The ratio of P_i to glucose-1-P has been estimated at about 3 : 300 (Heber and Santarius, 1965; Bassham and Krause, 1969), and subsequent studies agree with this ratio. The K_m values measured for glucose-1-P are one to two orders of magnitude higher than the glucose-1-P concentration calculated for the whole cell. Thus, although the phosphorylase activity, when tested at saturating concentrations of substrate, appears to be higher than starch synthase, in physiologic conditions this may not be the case. Still, it is possible that in conditions favoring starch synthesis,

and in the site of starch synthesis (the chloroplast or amyloplast), concentrations of P_i may be lower and concentrations of glucose-1-P may be higher than the whole-cell concentrations averaged over time.

One approach that would be useful in finding the physiologic role(s) of the starch phosphorylase would be the expression of antisense RNA in an organ such as the potato tuber [as Müller-Röber and colleagues (1992) have done for the AdPGlc PPase; see the following], followed by measurement of the corresponding enzyme (plastidial or cytosolic), and a thorough study of the effects (if any) of the consequence deficiency of the amyloplast phosphorylase on the amount and structure of the starch formed.

IV. ALTERNATIVE PATHWAYS

In studies reviewed previously (Preiss and Levi, 1980; Preiss, 1988), no relationship was noted between starch synthesis and UDPglucose pyrophosphorylase (UDPGlc PPase) (reaction 3b) activity. The high K_m values of starch synthase for UDPglucose, as compared to the measured cellular levels, strongly argue against a significant role for UDPGlc PPase in starch synthesis. It would seem that the major part, if not all, of UDPGlc PPase activity is localized in the cytosol and not in the organelle involved in starch synthesis—the amyloplast (Bird *et al.,* 1974; Robinson and Walker, 1979; Macdonald and ap Rees, 1983). Thus, this high activity does not appear to be localized where active starch synthesis occurs. In contrast, the maximum activities of ADPGlc PPase and ADPglucose-starch synthase, which are localized in the amyloplasts, are at least three times greater than the rate of starch accumulation in soybean cultures and 1.3 to 2.7 times greater than the rate of starch accumulation in the developing club of the spadix of *Arum* maculatum (ap Rees *et al.,* 1984).

An alternate pathway for starch synthesis has been proposed, which is based on the finding of a putative ADPGlc translocator in the envelope of both amyloplasts and chloroplasts. Akazawa *et al.* (1991) proposed that ADPglucose is synthesized in the cytosol by the sucrose synthase (rather than in the plastid by the action of the ADPGlc PPase, as is widely accepted), and is then transported into the plastid where it is converted into starch by the starch synthase. A critique of this hypothesis is presented in the chapter, "The Site of Starch Synthesis in Nonphotosynthetic Plant Tissues: The Amyloplast," where metabolite transport into the plastids is discussed, but it is worth mentioning here that this pathway does not fulfill the criteria mentioned in the preceding—that is, the experimental evidence does not support this alternative pathway.

Conversely, a large body of evidence strongly indicates that the main, if not the only, pathway of starch synthesis consists of the enzymatic reactions catalyzed respectively by ADPGlc PPase (reaction 3a), the starch synthase reaction (reaction 2), and the branching enzyme (reaction 5). The data supporting this view are from a number of biochemical and genetic studies.

V. RATE OF STARCH SYNTHESIS VERSUS ACTIVITIES OF THE STARCH BIOSYNTHETIC ENZYMES

A direct relationship between the increase in the activities of starch synthase and ADPGlc PPase, and the rate of starch accumulation, has been reported for developing maize endosperm (Ozbun *et al.*, 1973), wheat grain (Moore and Turner, 1969; Turner, 1969), and potato tubers (Sowokinos, 1976) (for a review of these, see Preiss and Levi, 1980).

The man-made intergenic hybrid, triticale (X-*Triticosecale* Wittmarck), may produce in development either plump or shriveled seeds (Dedio *et al.*, 1975). The difference between the two seeds was originally postulated to be due to higher amylase content. However, studies in which the activities of the starch biosynthetic enzymes and amylase were measured during seed development showed that even though the shriveled seeds contained more amylase than the plump seeds, the shriveled appearance occurred earlier than the increase in amylase activity (Ching *et al.*, 1983). Starch synthase and ADPGlc PPase activities, extracted at different stages of the seed development, were in excess of the measured rates of starch accumulation, indicating that these enzymes could play an important role in starch synthesis.

Similar results were obtained with germinating seeds of *Ricinus communis* (Reibach and Benedict, 1982). Starch levels increased about two-fold in the imbibed seed in 5 days. The starch synthase and ADPGlc PPase activities increased to a maximum 4 to 5 days after germination and were high enough to account for the observed rates of starch synthesis. In this study, the ADPGlc PPase activity was 1000-fold higher than the previously reported UDPGlc PPase activity (Nishimura and Beevers, 1979).

In maize ears, the apical kernels develop and pollinate several days after basal kernels; the kernels that are formed earlier may have higher survival probability, longer growth duration, and higher growth rates. Ou-Lee and Setter (1985b) compared the activities of the starch biosynthetic enzymes in the apical and basal kernels during development of synchronously pollinated ears. During the period of maximal starch synthesis, the ADPGlc PPase and starch synthase activities could account for the observed starch accumulation rate in basal kernels, but were slightly less than adequate to account for starch synthesis in apical kernels. It should be pointed out,

however, that it was later shown (Plaxton and Preiss, 1987) that during extraction, maize endosperm ADPGlc PPase is particularly sensitive to endogenous protease activity, which significantly changes the regulatory properties of the enzyme and decreases its stability. It is therefore possible, as Ou-Lee and Setter (1985b) suggested, that their assays may have underestimated the starch biosynthetic enzyme activities but, nevertheless, a rough correlation between maximal starch accumulation and the levels of the starch biosynthetic enzymes was noted. In an extension of this study, the effect of differential temperature increases on the growth rate and size of the apical kernels was examined (Ou-Lee and Setter, 1985a). The temperature was increased to 25°C at 7 days after pollination, as opposed to the lower temperatures normally experienced by the plant at nights and in cool weather. The tip-heated treatment slightly increased the size of the apical kernels at the expense of slightly decreasing the size of more numerous basal and middle position kernels, and some of the developmental events in the apical kernels were accelerated. The maximal levels of ADPGlc PPase and starch synthase activities occurred earlier and correlated well with the earlier rise of starch levels in the heated apical kernels, and the ADPGlc PPase activities were sufficient to account for the starch content measured. Starch synthase activities, measured as granule-bound enzymes, were insufficient. Soluble starch synthase activity was not measured.

Addition of adenine (0.1 m*M*) to cultures containing tobacco callus cells increased the starch content almost 4-fold in 3 days (Gamanetz and Gamburg, 1981). Addition of other purine or pyrimidine bases had no effect on the starch content. In the cells grown with adenine, there was a 100-fold increase in the ADPglucose content (there was no effect on the UDPglucose content) and a 2.5-fold increase in the specific activity of ADPGlc PPase. Adenine has been shown to increase the starch content of other plant cells in suspension cultures (i.e., soybean, potato, *Atriplex* sp., dewberry) (Gamanetz and Gamburg, 1981), and it was concluded that in the plant cells the adenine pool may be limiting for ADPglucose synthesis. Addition of adenine would stimulate ADPglucose synthesis and, therefore, starch synthesis.

In a series of experiments to determine which sugar nucleotide—ADPglucose or UDPglucose—plays the major role in starch synthesis in nonphotosynthetic plant cells, ap Rees *et al.* (1984) estimated the in vivo rates of starch synthesis in the developing club of the spadix of *Arum maculatum* and in suspension cultures of soybean. They compared these estimates with the maximum catalytic activities of four enzymes: ADPglucose- and UDPglucose–starch synthase, ADPglucose pyrophosphorylase, and UDPglucose pyrophosphorylase; the amounts of ADPglucose and UDPglucose in these cells were also determined. The conclusion was that

in *Arum* clubs and soybean cultures, starch synthesis proceeds almost entirely via ADPglucose.

VI. A MISSING STEP?

As discussed previously, only three reactions—those catalyzed by ADPGlc PPase, starch synthase, and branching enzyme—are needed to synthesize all the glucosidic linkages found in the starch granule. However, it is interesting to note that the *sugary* (*su*) 1 mutation in the maize endosperm does not affect the expression of the genes of any of the three activities, but still results in a significant reduction in starch granule formation. This decrease in starch accumulation is accompanied by an increase in the content and an increase in the content of a water-soluble α-1,4-glucan phytoglycogen in such a way that the total polysaccharide content approaches that of normal maize. Thus, another enzyme activity may be required to complete the formation of the starch granule. Pan and Nelson (1984) showed that maize endosperm displaying the *su* 1 mutation was defective in debranching enzyme activity. More recently, the *su* 1 was cloned (James *et al.*, 1995), and sequence analysis of its cDNA showed that it has a high degree of homology with a bacterial isoamylase (Yang *et al.*, 1996). Thus, Ball *et al.* (1996) proposed that the *su* 1 gene, believed to be the structural gene for isoamylase activity, is required for formation of the finished amylopectin product. In other words, a fourth enzyme would be needed to convert the product of the branching enzymes into amylopectin, which is able to crystallize, trapping the amorphous amylose to form the starch granule. This subject is discussed more extensively in the chapter, "Open Questions and Hypotheses in Starch."

VII. SUMMARY

The major route to starch biosynthesis involves three reactions. The first reaction, catalyzed by ADPGlc PPase (glucose-1-P adenylyltransferase; EC 2.7.7.27), results in the synthesis of the glucosyl donor ADPglucose. The second reaction, catalyzed by starch synthase (ADPglucose: 1,4-α-D-glucan 4-α-D-glucosyltransferase; EC 2.4.1.21), transfers the glucosyl group of ADPglucose to the nonreducing end of an α-1,4-glucan primer to form a new α-1,4-glucosidic bond. The synthesis of the α-1,6-branch linkages found in amylopectin is catalyzed by branching enzyme (1,4-α-D-glucan: 1-4-α-D-glucan 6-glycosyl-transferase; EC 2.4.1.18).

The kinetic properties of the enzymes in the ADPglucose pathway (K_m and V_{max} values), together with the concentrations of substrate and effector metabolites in plant cells, are consistent with a major role for the pathway in starch synthesis. Conversely, the properties of the UDPglucose-specific starch synthases and starch phosphorylases (i.e., the high K_m values for their substrates, UDPglucose and glucose-1-P, respectively), as compared to the concentration in the relevant cellular compartments, argue against a significant role or UDPglucose starch synthase and starch phosphorylase in starch biosynthesis. No relationship has been observed between starch synthesis and the activities of starch phosphorylase or UDPGlc PPase in the tissues studied. Moreover, in some starch-synthesizing plant tissues, the synthesis of UDPglucose only occurs in the cytosol and not in the amyloplast, where starch is made.

Analyses of the starch biosynthetic system in a number of plants and green algae indicate that an important site of regulation of starch synthesis is at the ADPGlc PPase and that 3PGA and P_i are important regulatory metabolites of that enzyme.