Plant Physiol. (1995) 107: 673–677

Update on Metabolic Regulation

What Controls the Amount and Structure of Starch in Storage Organs?

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The regulation of starch synthesis in the starch-storing organs of higher plants—organs such as tubers, the embryos of grain legumes, and the endosperms of cereals, in which starch constitutes 50 to 80% of the dry weight at maturity—is complex and poorly understood. Our ignorance of this process has been highlighted by the recent upsurge of interest in starch synthesis, fueled largely by the possibility of producing novel starches by genetic modification. Details of the metabolic pathway itself remain the subject of controversy, and remarkably little is known of the factors that determine either the rate of synthesis or the structure of the starch in storage organs. Some widely accepted views about the regulation of starch synthesis are no longer tenable or require revision in the light of recent findings. We shall discuss briefly the nature of the pathway of starch synthesis, then examine critically the current ideas about factors that determine the flux through the pathway and two important aspects of starch structure: the ratio of amylose to amylopectin and the branching of amylopectin.

THE NATURE OF THE PATHWAY

Evidence for the pathway outlined in Figure 1 has been discussed in detail (Preiss, 1988; Okita, 1992), and this remains the model that best fits the published data. To understand the regulation of the pathway, three steps in particular require further characterization. First, the precise route by which carbon enters the amyloplast has not been established for many storage organs. The idea that carbon may enter as ADPG is not tenable at present. There is abundant evidence that ADPG pyrophosphorylase is required for starch synthesis and that the activity of this enzyme is plastidial (Okita, 1992). It is likely that carbon for starch synthesis enters the amyloplast as hexose phosphate. Amyloplasts from developing pea embryos and wheat endosperm are capable of converting exogenously supplied hexose phosphate to starch at physiologically meaningful rates. In the former case the process is specific for Glc-6-P, whereas in the latter case Glc-1-P is strongly preferred (Hill and Smith, 1991; Tetlow et al., 1994). Transporters in the amyloplast envelope probably catalyze a hexose-phosphate/phosphate exchange, but none has been fully characterized.

The other two steps requiring further characterization are those catalyzed by starch synthase and starch-branching enzyme. Both enzymes exist as multiple isoforms, and starch synthase isoforms may be either soluble or tightly bound to starch granules. However, for most storage organs a full identification of the proteins responsible for these two enzyme activities has not been achieved. Some of the problems presented by our lack of knowledge of these enzymes to studies of regulation will be described below.

Although there is no unequivocal evidence for the involvement in starch synthesis of enzymes other than those shown in Figure 1, this remains a possibility. Two areas where current research may cause a revision of our model of the pathway are the mechanism of initiation of starch polymers and the roles of enzymes that degrade or modify starch polymers, for example debranching and disproportionating enzymes and starch phosphorylase.

THE CONTROL OF FLUX

It is widely accepted that most of the control of flux through the pathway of starch synthesis in storage organs rests with ADPG pyrophosphorylase (e.g. Stark et al., 1992), but definitive evidence for this is lacking. The three main pieces of evidence cited to support this view are as follows.

First, the enzyme has the hallmarks of a so-called "pacemaker" or "rate-limiting" reaction. It is the first committed enzyme on the pathway of starch synthesis, it is believed to be effectively irreversible in vivo, and it has pronounced allosteric properties. However, in spite of its persistence in the literature, the idea that there are "pacemaker" enzymes identifiable by their properties and position in metabolic pathways has been discredited (for review, see ap Rees and Hill, 1994). The control of flux is partitioned between all of the enzymes on a pathway, in a manner that is likely to differ from one type of organ to another, with environmental conditions, and with developmental age.

Second, alteration of the activity of ADPG pyrophosphorylase in storage organs changes the amount of starch that they accumulate. In the endosperm of maize carrying mutations at the *sh2* or *bt2* locus (which encode large and small subunits of the enzyme, respectively), in the embryos of peas carrying a mutation at the *rb* locus, and in the tubers

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Abbreviations: ADPG, ADP-Glc; *amf*, *amylose-free*.

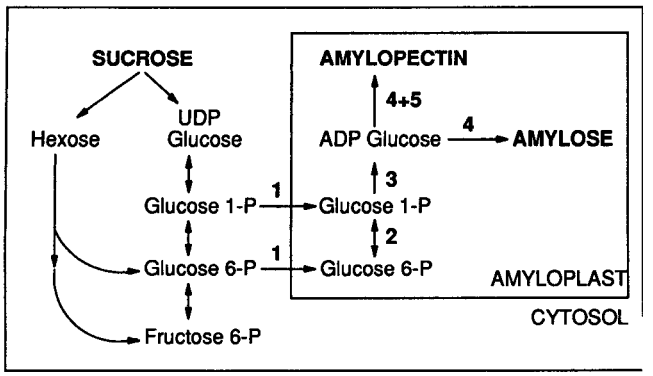

Figure 1. The pathway of starch synthesis in nonphotosynthetic, starch-storing organs. Suc enters the cell and is metabolized via glycolysis in the cytosol. A hexose phosphate crosses the amyloplast envelope. Inside the amyloplast, ADPG is synthesized via ADPG pyrophosphorylase. ADPG is the substrate for the synthesis of the starch polymers amylose and amylopectin via isoforms of starch synthase and starch-branching enzyme. 1, Hexose-phosphate translocator; 2, plastidial phosphoglucomutase; 3, ADPG pyrophosphorylase; 4, starch synthase; 5, starch-branching enzyme.

of potatoes transformed with an antisense construct for the large subunit of the enzyme, large reductions in enzyme activity result in lower starch contents (Müller-Röber et al., 1992; Okita, 1992). In the tubers of transformed potatoes expressing the single gene encoding ADPG pyrophosphorylase from *Escherichia coli*, starch content is increased (Stark et al., 1992). This general relationship between the activity of ADPG pyrophosphorylase and the accumulation of starch has been taken as evidence that the enzyme controls the rate of starch synthesis. However, although it certainly tells us that the enzyme is involved in starch synthesis, it gives no indication of the importance of the enzyme in controlling flux relative to other enzymes on the pathway. In pea embryos, for example, a reduction in the activity of starch-branching enzyme caused by a mutation at the *r* locus also reduces the rate of starch synthesis (Burton et al., 1995). Without a quantitative analysis of relationships between the activities of ADPG pyrophosphorylase and starch-branching enzyme and the rate of starch synthesis during embryo development, it is not possible to determine which of these two enzymes is the more important in controlling flux or whether either is of overriding importance in this respect.

Third, an important role for ADPG pyrophosphorylase in storage organs has been deduced from the fact that it is undoubtedly important in determining the rate of starch synthesis in leaves. Quantitative measurements of the relationship between the rate of chloroplastic starch synthesis and the activity of ADPG pyrophosphorylase, utilizing a mutant *Arabidopsis* with dramatically reduced levels of the enzyme, show that this enzyme is much more important in controlling the rate of starch synthesis than other enzymes on the pathway from hexose phosphates to starch in the chloroplast (Neuhaus and Stitt, 1990). There is little reason, however, to assume that this is also the case in nonphotosynthetic starch-storing organs; in fact, the photosynthetic and nonphotosynthetic pathways are likely to be regulated differently. Photosynthetic starch synthesis must respond in a rapid, sensitive, and modulated manner to changes in the balance between the rate of carbon assimilation of the leaf and the rate of Suc synthesis and export to the rest of the plant, whereas starch synthesis in a storage organ probably proceeds at a relatively steady rate over long developmental periods. The ADPG pyrophosphorylase of leaves and storage organs may have different subunit compositions and regulatory properties. The enzymes from barley leaf and endosperm, for example, differ in both of these respects (Kleczkowski et al., 1993).

Recent work suggests that the soluble activity of starch synthase may be more important than ADPG pyrophosphorylase in controlling the rate of starch synthesis in storage organs (Jenner et al., 1993; Keeling et al., 1993). This idea arises from experiments in which the very unusual thermal stability properties of starch synthase have been exploited to alter its activity in vivo. The soluble activity from all of the sources so far examined is inactivated at temperatures of 37 to 45°C and above. Brief treatment of wheat ears and grains at elevated temperatures decreases soluble activity and the rate of starch synthesis to a remarkably similar degree, suggesting that this activity is very important in regulating starch synthesis (Jenner et al., 1993; Keeling et al., 1993). There is speculation that the pronounced decrease in the yield of starch-storing organs observed at elevated temperatures may be due primarily to decreased activity of starch synthase. However, more information is needed about the effects of heat treatment before firm conclusions can be drawn. Although heating does not appear to affect several other soluble enzymes involved in starch synthesis (Jenner et al., 1993; Keeling et al., 1993), by no means all of the enzymes on the pathway from Suc to starch have been examined, and the effects on, for example, the permeability of the amyloplast membrane are not known.

The question of the control of flux through the pathway of starch synthesis in storage organs remains open. We suggest that its resolution lies in the application of metabolic control analysis, in which the relationship between the activity of an enzyme and the flux is used as a measure of the importance of the enzyme in controlling that flux (ap Rees and Hill, 1994). Although this analysis can be theoretically and practically difficult, we believe that a systematic and quantitative approach to the control of flux is urgently required to resolve the contradictions that have arisen from nonquantitative or incomplete analyses. Quantitative information will also allow a rational approach to the biotechnological modification of starch content.

THE CONTROL OF STRUCTURE

Starch exists as semicrystalline granules composed of two Glc polymers: amylopectin, a highly branched structure of relatively short chains of α1,4-linked Glc residues joined by α1,6 linkages, and amylose, a much smaller and relatively unbranched polymer of long chains of α1,4-linked Glc residues. Amylopectin makes up 70 to 80% of most starches. Within the starch granule, the amylopectin molecules are radially arranged with their free, nonreduc-

ing ends pointing outward. The branches of the amylopectin molecules form clusters at intervals of about 9 nm along the long axes of the molecules. Adjacent branches within clusters form double helices, which pack together to give crystalline arrays. The amylose component of the granule probably exists in an amorphous state, but its location relative to the amylopectin crystallites is not fully understood. Superimposed upon this molecular structure is a periodicity—thought to reflect diurnal variation in some aspect of synthesis—in the degree of crystallinity, such that the granule consists of concentric, alternate shells of greater and lesser crystallinity (reviewed by Smith and Martin, 1993).

The way in which starch structure is determined remains the least understood aspect of starch synthesis. It is generally accepted that granule growth occurs in a zone at the surface and that the synthesis in this zone of two different kinds of polymers is a function of the existence of multiple isoforms of starch synthase and starch-branching enzyme with different properties and spatial locations. For the most part, the mechanisms determining polymer structure and packing are unknown. We shall consider two aspects of structure for which mechanisms have been suggested: the synthesis of amylose and the branching pattern of amylopectin.

The Synthesis of Amylose

The only aspect of the determination of starch structure about which there is unequivocal information at a biochemical level is the synthesis of amylose. This has been a focus of interest because of the existence of mutations that eliminate amylose from storage starch. These mutations—at the *waxy* loci of cereals and the *amf* locus of potatoes—lie in genes encoding proteins that are exclusively bound to starch granules. Highly conserved proteins of this class (referred to collectively as Waxy proteins) are found in all storage organs examined thus far. The mutations eliminate not only the amylose component of the granule but also most of the granule-bound starch synthase activity. This indicates that the Waxy proteins are granule-bound starch synthases, exclusively responsible for the synthesis of amylose (Preiss, 1988; Smith and Martin, 1993). Further confirmation of the role of these gene products in the synthesis of amylose has come from experiments with potatoes, in which the *amf* mutant has been complemented by expression of the wild-type *amf* gene (van der Leij et al., 1991).

The granule-bound nature of the proteins responsible for amylose synthesis has generated proposals to explain how almost unbranched amylose can be synthesized at the same time as highly branched amylopectin. It is proposed that soluble starch synthase and starch-branching enzyme act together at the periphery of the granule to synthesize amylopectin. The amylopectin crystallizes to form the granule matrix, to which the Waxy protein binds. This protein then synthesizes a polymer that, because of its location within or at the surface of the crystalline matrix, is unavailable to starch-branching enzyme and thus remains unbranched (Denyer et al., 1993; Smith and Martin, 1993). This is an appealing model, but two recent discoveries suggest that it is simplistic and requires revision. These are the existence of other granule-bound starch synthases and granule-bound starch-branching enzyme and the distribution of amylose within starch granules of reduced amylose content.

The existence on starch granules of isoforms of starch synthase other than the Waxy class is probably a widespread phenomenon. The endosperms of maize and wheat and the embryo of pea all have granule-bound isoforms of starch synthase in addition to the Waxy protein (Macdonald and Preiss, 1985; Denyer et al., 1993, 1995). The model for the synthesis of amylose predicts that, because of their location, these non-Waxy granule-bound starch synthases will contribute to amylose rather than amylopectin synthesis. However, there is at present no evidence for this. The starch of the *waxy* mutant of maize is amylose free even though it retains non-Waxy starch synthase activity (Macdonald and Preiss, 1985).

Two suggestions for the roles of these isoforms are as follows. First, they may interact with granule-bound starch-branching enzymes to contribute to amylopectin synthesis. Although branching enzyme is generally regarded as soluble, we have found that much of the activity of this enzyme is tightly bound to starch granules in pea embryos and wheat endosperm (Denyer et al., 1993, 1995). Its existence creates a considerable problem for the model of the synthesis of amylose. For the synthesis of unbranched polymers to occur, an active, granule-bound branching enzyme would have to be in a different location on the granule from the Waxy protein. Second, both the non-Waxy granule-bound starch synthases and the granule-bound branching enzymes may be isoforms active in the soluble fraction of the amyloplast that have become trapped within the amylopectin matrix. There is some evidence to support this suggestion. In developing pea embryos the major soluble isoform of starch synthase and the two soluble isoforms of starch-branching enzyme are almost certainly the same proteins as the granule-bound non-Waxy starch synthase and the two granule-bound branching enzymes, respectively (Denyer et al., 1993). Wheat and maize endosperms also have isoforms of starch synthase that are both soluble and granule bound (Mu et al., 1994; Denyer et al., 1995). It is tempting to speculate that soluble starch synthases and branching enzymes become fossilized within the granule matrix as the amylopectin they produce crystallizes around them. Whether these isoforms remain active within the granule, and the nature of their products if they do, remains unknown.

Recent examination of the distribution of amylose within starch granules of reduced amylose content has produced surprising results that will necessitate a revision of the model for the synthesis of amylose. Visser and colleagues have used iodine staining to examine the starch granules of potatoes in which the amount of amylose has been reduced by introduction of an antisense construct for the *amf* gene (Kuipers et al., 1994). Rather than staining uniformly, these granules stain red with a blue core, indicating that the amylose content of the inner region is far higher than that

of the periphery. The reasons for this unexpected distribution of amylose are unknown, but this result clearly illustrates that our understanding of amylose synthesis is far from complete.

The Branching of Amylopectin

Two distinct views about the determination of the branching pattern of amylopectin have been put forward over the last decade: that the pattern represents a balance between the activities of branching and debranching enzymes, and that the pattern can be explained largely by the properties of branching enzymes. There is insufficient experimental evidence at present to allow either to be unequivocally accepted or rejected.

The view that the structure of amylopectin is determined by both branching and debranching enzymes comes from studies of the effects of mutations at the *su1* (*sugary-1*) locus of maize. Although the endosperm of mutant plants produces some normal starch, much of its α1,4, α1,6-linked glucan is in the form of a very highly branched, soluble polymer known as phytoglycogen. The mutations alter the activities of several enzymes of starch metabolism, including both starch-branching enzyme and debranching enzyme (Preiss, 1988; Doehlert et al., 1993). The effects on debranching enzyme are particularly pronounced and have led to the suggestion that this enzyme is involved in the normal synthesis of amylopectin. It is proposed that the structure of amylopectin is determined by a balance between the actions of branching and debranching enzymes. A decrease in the activity of debranching relative to branching enzyme would then result in an abnormally highly branched polymer, as seen in the *su1* mutant (Pan and Nelson, 1984). This suggestion is a radical departure from the conventional view that starch synthases and starch-branching enzymes determine the structure of amylopectin and that debranching enzyme is involved in starch degradation. A serious assessment of the validity of this suggestion must await further information about, for example, the molecular basis of the *su1* mutations and the location of active debranching enzyme in the developing endosperm.

The conventional view that the branching pattern of amylopectin is determined by the properties of starch-branching enzyme is supported by recent work on isoforms of this enzyme. All of the isoforms for which full amino acid sequences have been predicted can be divided into two classes, A and B, which differ in distinct ways. The storage organs for which more than one isoform has been described—which include rice and maize endosperm and pea embryos—possess one isoform of each class (Burton et al., 1995). Speculation that A and B isoforms may play distinct roles in determining the branching pattern of amylopectin is supported by analysis of the products of these isoforms purified from maize endosperm (Takeda et al., 1993). The two isoforms differ considerably in their affinities for substrates with different degrees of branching. When allowed to branch amylose, the class A isoform (maize BEII) transfers chains that are on average shorter than those transferred by the class B isoform (maize BEI). Evidence that these differences may be of biological significance is provided by parallel changes in the isoform complement and the structure of amylopectin in the developing pea embryo (Burton et al., 1995). The branching enzyme activity during the first part of embryo development is accounted for by the class A isoform (pea BEI), whereas later in development both class A and B isoforms are present. The iodine-binding characteristics of amylopectin in the embryos change through development in a manner that suggests that average branch length increases. This change in amylopectin structure is precisely that expected if the properties of the branching-enzyme isoforms, as defined for maize, are primary determinants of branch length.

FUTURE PROSPECTS

Although our understanding of the regulation of starch synthesis in storage organs is at a rudimentary level, there are real prospects for progress in the near future. Increasing appreciation of the power of metabolic control analysis combined with the availability of plants with altered activities of specific enzymes will facilitate our understanding of the control of flux. Combined approaches in which the study of enzymes of starch synthesis is related to the chemistry of starch polymers and granules offer the best prospects for understanding starch structure. Studies such as those of Preiss and colleagues on the products of starch-branching enzymes provide new insight and direction in this field (Takeda et al., 1993). Here, too, the availability of plants with altered activities of specific enzymes is important. For example, novel information about the synthesis of amylose will be gained from potato tubers with reduced levels of the Amf protein (Kuipers et al., 1994).

Better understanding of the regulation of starch synthesis will also require more research into the basic biochemistry of the pathway. We suggest that two areas in particular need research and consideration. First, the picture of the isoforms of starch synthase and starch-branching enzyme is incomplete for almost all storage organs. We have even less idea about the nature and roles of other starch-modifying enzymes that may determine structure, such as debranching enzyme and phosphorylase. We cannot hope to explain the regulation of starch synthesis until all of the proteins involved are identified. A great deal more research on the location and isoform composition of these enzyme activities is needed. Second, we must anticipate that the regulation of starch synthesis will differ from one type of storage organ to another and throughout the development of the organ. Endosperms, embryos, and tubers are of different developmental origins, and the factors that determine the rate at which they receive carbon and the way in which carbon is partitioned within them are probably different. The structure of starch differs among these types of organs, so the factors that determine structure must also differ. In at least some storage organs there are major changes in the nature of the pathway of starch synthesis during development. In pea embryos, for example, the ratios of the two major isoforms of starch synthase and of starch-branching enzyme change radically through development (Dry et al., 1992; Burton et al., 1995). Such

changes in isoform composition are likely to be responsible for the widely observed changes throughout development in the structure of newly synthesized starch (Shannon and Garwood, 1984).

ACKNOWLEDGMENT

We thank Professor Mike Emes and colleagues, University of Manchester, UK, for making unpublished data available to us.

Received October 6, 1994; accepted November 17, 1994.

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