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Update on Biochemistry

Recent Progress toward Understanding Biosynthesis of the Amylopectin Crystal[1]

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Plant starch granules provide the largest percentage of calories in the human diet. Starch consists almost entirely of the Glc homopolymers amylopectin and amylose. Amylopectin is the major contributor to both mass and granule structure. Because of the very basic role that starch plays in our society, increased understanding of the biosynthetic mechanisms that produce amylopectin is important. Amylopectin is an important industrial raw material, and much remains to be determined about the relations between its biosynthesis and functionality. It is also a fascinating molecule in its own right, because it exhibits a high degree of architectural specificity despite containing only one type of monomeric unit (i.e. the glucosyl group) connected via just two linkage types. Thus, significant insights into basic biochemical mechanisms may be obtained by studying amylopectin biosynthesis.

Amylopectin is chemically similar to glycogen in that both are $\alpha(1\rightarrow4)$-linked, $\alpha(1\rightarrow6)$-branched Glc homopolymers, although a major difference between them is the organization of the latter into large, insoluble, semicrystalline granules. Polysaccharides of this type synthesized in vitro do not readily crystallize, which is indicative of the relation between amylopectin biosynthesis and granule formation. In this *Update* we address the biological mechanisms underlying amylopectin crystallization, in particular those steps that could distinguish the amylopectin pathway in plants from the glycogen pathway in a presumptive cyanobacterial ancestor. The enzymes to be discussed include starch synthases (SSs) and starch branching enzymes (BEs), both of which are involved directly in amylopectin biosynthesis. In addition, we discuss two enzymes for which potential roles in amylopectin biosynthesis are suggested by genetic data, namely starch debranching enzymes (DBEs) and disproportionating enzyme (D-enzyme). Other important aspects of amylopectin biosynthesis that are outside the scope of this *Update* include production of the glucosyl unit donor ADPGlc, amylose biosynthesis (Ball et al., 1998), polymer chain initiation, and granule initiation.

ELEMENTS OF AMYLOPECTIN STRUCTURE

We begin with a brief discussion of amylopectin structure to describe the end product and define important questions regarding the biosynthetic mechanism (for more comprehensive reviews, see Hizukuri et al., 1989; Gallant et al., 1997, and refs. therein). The chemical nature of amylopectin is straightforward: linear chains of $\alpha(1\rightarrow4)$-linked glucosyl units are joined to each other by $\alpha(1\rightarrow6)$ branches (Fig. 1A). A chains participate in branch linkages only through their reducing end and thus are unbranched; in contrast, B chains are branched because they are linked to other chains through the C_6 of one or more glucosyl units. Approximately 5% of the residues participate in both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages.

Amylopectin exhibits hierarchical levels of specific architectural structure. At the 0.1- to 1.0-nm scale, i.e. within individual chains, structure is described by branch location and chain length. Amylopectin can be completely converted into linear chains by enzymatic cleavage of all branch linkages, and the frequency of chains of specific length can then be quantified (Morell et al., 1998, and refs. therein). Such data reveal non-random chain length distributions generally conserved in amylopectin from different plants. Other studies involving enzymatic treatment prior to determination of residual chain length distribution provide estimates of the distances between branch points. This parameter also is non-random, indicating that branches are clustered. Thus, to understand the amylopectin biosynthetic mechanism, it is necessary to know what determines the specific lengths of A and B chains and how the non-random location of branch linkages along B chains is attained.

The next structural level occurs on the 10-nm scale (Fig. 1B). Regions called crystalline lamellae, recognizable by

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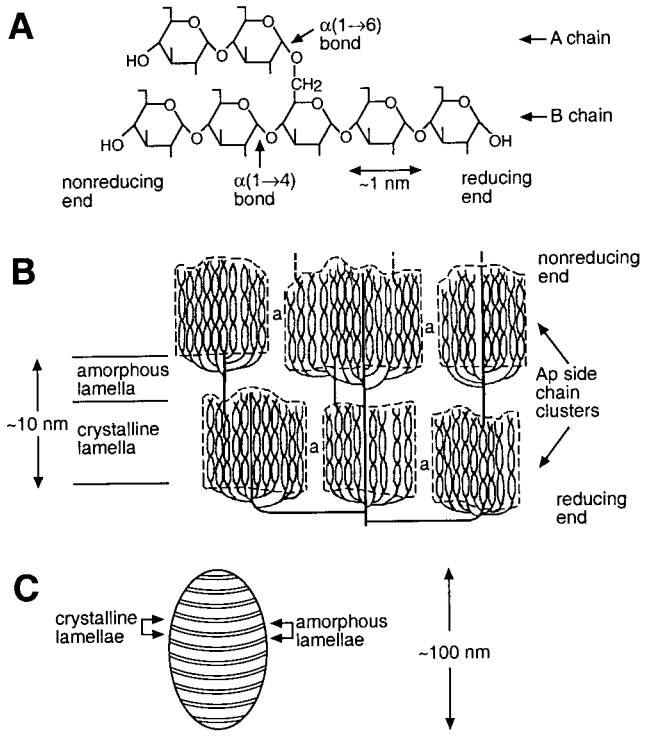

Figure 1. Diagrammatic representation of the proposed first three levels of amylopectin (Ap) structure (adapted from Gallant et al., 1997). A, Connection of glucosyl units via α(1→4) and α(1→6) glycoside linkages. The specific chain organization shown is not intended to illustrate the actual structure of amylopectin. B, Cluster model of amylopectin structure. Solid lines indicate glucan chains, and intersections between them indicate branch linkages. Dotted lines indicate the boundaries of amylopectin side chain clusters in which primarily unbranched chains associate in tightly packed double helices. a, Amorphous areas that separate amylopectin side chain clusters. C, Diagrammatic representation of a blocklet, which is made up of amylopectin side chain clusters organized into a discrete unit.

their resistance to acid hydrolysis, are thought to comprise tightly associated double helices containing primarily A chains and unsubstituted spans of B chains. Crystalline lamellae alternate with regions susceptible to acid hydrolysis called amorphous lamellae, which are thought to contain frequent branch linkages and thus be less densely packed. The 9- to 10-nm thickness of the repeating unit of crystalline and amorphous lamellae is generally conserved in the plant kingdom (Jenkins et al., 1993). Crystalline lamellae are thought to be divided into discrete units about 10-nm wide called amylopectin side chain clusters (Gallant et al., 1997). Clustering of branches in the lowest order structure is thought to allow organization of the subsequent level, because regions of relatively low branch frequency are necessary for amylopectin side chain clusters to form. Specific chain length distributions and arrangements may also be required for side chain cluster formation to be energetically favorable.

The next level of structure, on the 100-nm scale, is proposed to be the blocklet (Gallant et al., 1997). Crystalline and amorphous lamellae were seen by transmission electron microscopy to be grouped into discrete, elongated structures surrounded by relatively large, non-crystalline regions (Fig. 1C). Areas between blocklets are about 10 to 20 nm wide, compared with 3 to 4 nm for amorphous lamellae. Blocklets within a granule vary in size and shape, but can be approximated as oblate spheroids with short diameters of 20 to 500 nm. Blocklets are believed to be aligned relative to each other in various ways, rather than having a common orientation.

The next structural level within starch granules is observed as characteristic alternating rings ranging in thickness from 120 to 500 nm. These distinct, alternating rings are called crystalline shells and semicrystalline shells. Different shells may each consist of distinct types of blocklets that vary in size and/or packing geometry. Considering their dimensions, two or three layers of blocklets could make up a shell. Further considerations of starch granule structure involve packing of amylose and other components such as lipids into the framework established by amylopectin.

Amylopectin biosynthesis must be examined within the framework of the overall hierarchical structure of the product. Although the basic enzymatic steps are relatively well understood, much remains to be learned about the relationship between enzyme action and amylopectin structure. The SSs, BEs, and, potentially, DBEs and D-enzymes, will determine the fundamental structure of the molecule, e.g. A chain length and the placement of branches along B chains. At some point the glucans produced by the enzyme system must assume higher order structures, although neither how nor when this occurs is known. For example, in a minimalist model it is possible that each A chain as it is synthesized is packaged immediately into a side chain cluster. Alternatively, clusters might not form until all of the constituent chains have been formed. Extending the latter suggestion to the other extreme, entire amylopectin molecules may be packaged all at once into amylopectin side chain clusters, alternating crystalline and amorphous lamellae, blocklets, and crystalline or semicrystalline shells. Thus, it is necessary to understand not only the particular activity of each enzyme, but also the relationship between the synthesis of the primary product and subsequent packing into higher order structures.

Consideration of amylopectin structure highlights an inherent difficulty in analyzing these biosynthetic enzymes, which is that their native substrates are not defined. SSs, BEs, and other potential biosynthetic enzymes act within plastids on glucans likely to be very different in terms of concentration, secondary structure, and tertiary arrangement than artificial substrates used in vitro. Any in vitro enzyme characterization must be interpreted relative to actual cellular conditions, and this consideration is particularly important regarding amylopectin biosynthesis.

ROLES OF SS AND BE

Two general views can be considered regarding which enzymes account for amylopectin structure. One possibility is that BEs and SSs together are entirely responsible for amylopectin biosynthesis, which requires that the product

of these enzymes is capable of attaining specific higher order structures. The alternative is that immature polymers produced by SSs and BEs are further modified to promote folding into mature amylopectin. Additional modifications are suggested by the effects of mutations seemingly unrelated to SS or BE function on amylopectin structure. The position of SSs and BEs within the general starch biosynthetic pathway is outlined in Figure 2, and some pertinent information about these enzymes is considered here (for detailed reviews, see Smith et al., 1997, and refs. therein).

SSs transfer glucosyl units from ADPGlc to the nonreducing ends of growing polymers via new $\alpha(1\rightarrow4)$ linkages (Fig. 3). Starch-producing tissues contain multiple SSs that appear to serve specific roles in the synthesis of amylose and/or amylopectin. At least four distinct SS isoforms can be defined based on sequence relationships (Cao et al., 1999; Li et al., 1999, and refs. therein). All plants possess the granule-bound isoform GBSSI, whereas the SSI, SSII, and SSIII isoforms, located partially or entirely in the soluble phase, are found in different combinations. For example, in vitro measurements suggest that the dominant activity in maize endosperm is SSI, in pea embryos SSII, and in potato tubers SSIII, even though each species have genes for all three isoforms. In wheat endosperm, SSI, SSII, and SSIII all are present in the soluble phase, and further complexity is evident because the abundance of SSII and its partitioning between the granule and soluble phases varies during grain development (Li et al., 1999).

The GBSSI, SSI, SSII, and SSIII isoforms are conserved broadly in evolution, suggesting that specific functions have been selected. Mutational and antisense analyses support this hypothesis, because interference with a specific isoform often results in structural alterations in amylopectin (Edwards et al., 1999; Lloyd et al., 1999, and refs. therein). Thus, in some instances, SSs do not substitute fully for one another. Details about the distinct enzymatic properties of SS isoforms are emerging through characterization of purified native or recombinant enzymes (Imparl-Radosevich et al., 1999, and refs. therein). Considering, however, that the native primers are not characterized and that isoform specificities overlap to varying degrees, it remains difficult to define individual SS functions. Isoform specificities, e.g. with respect to chain length, action in the proximity of branch points, and relative action on freely soluble versus granule-associated glucans, thus remain a challenging subject for research.

BEs also are present as multiple isoforms. BEs catalyze the cleavage of $\alpha(1\rightarrow4)$ linkages and transfer of the released

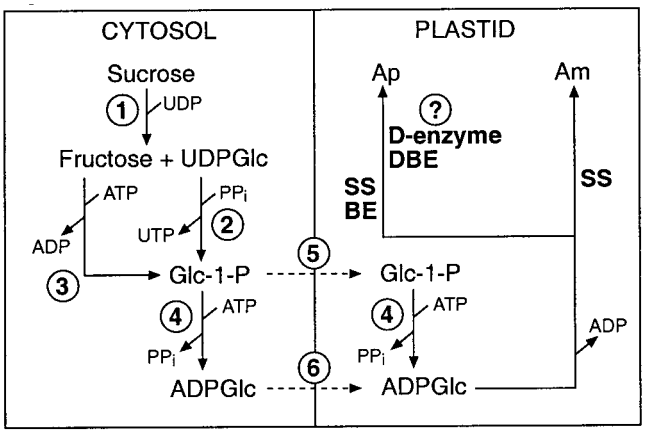

Figure 2. General pathway of starch biosynthesis beginning with Suc as the product of photosynthesis. Activities are as follows. 1, Suc synthase; 2, UDP-Glc pyrophosphorylase; 3, glycolytic enzymes including phosphoglucomutase; 4, ADPGlc pyrophosphorylase; 5, hexose phosphate transporters; 6, ADPGlc transporters. Not all plants possess both the indicated transporters and cytosolic ADPGlc pyrophosphorylase. Transport of Glc-1-P is depicted as a possible example and is not meant to imply that hexose phosphate transporters are necessarily specific for this molecule. Ap, Amylopectin; Am, amylose.

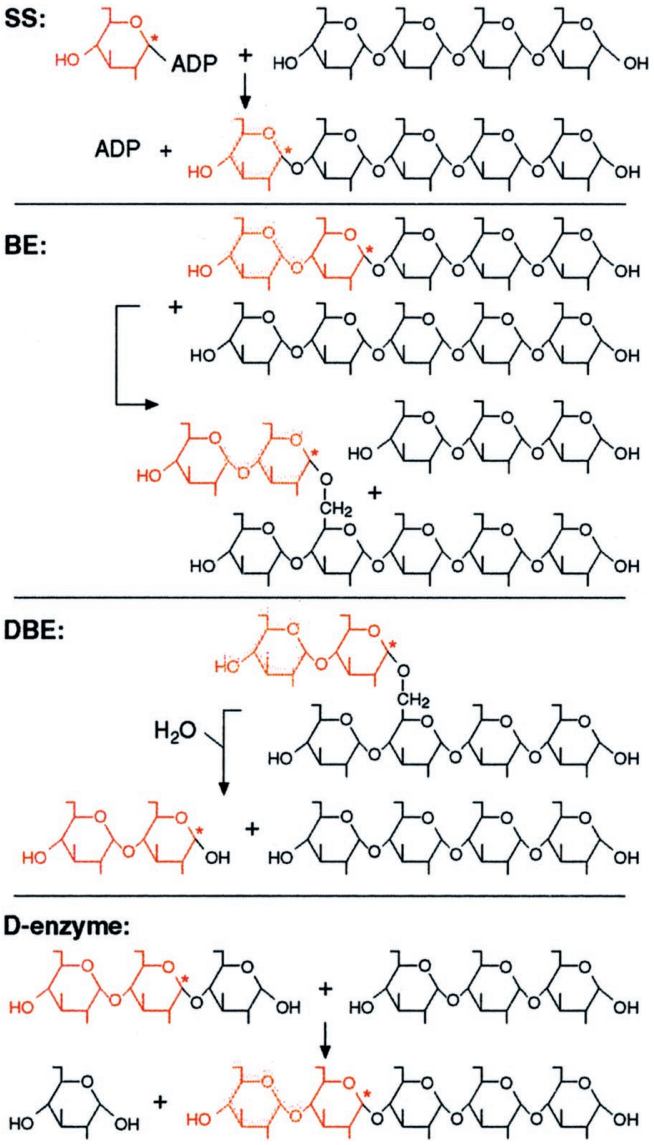

Figure 3. Diagrammatic representation of the chemical reactions catalyzed by enzymes involved in amylopectin biosynthesis. Donated glucosyl units are shown in red, and asterisks indicate reducing carbons through which these glucans are transferred. Structures are shown only to illustrate changes in linkage structure and are not intended to indicate substrate specificities.

reducing end to a C6 hydroxyl, creating a new $\alpha(1\rightarrow6)$ linkage (Fig. 3). Two classes are known from specific sequence conservation patterns (Smith et al., 1997). Two nomenclatures have evolved in which one class is known as BEI in maize or B in pea, and the other class as BEII in maize or A in pea. In maize, the BEII class contains two genetically distinct members, BEIIa and BEIIb. Pea embryos, potato tubers, and endosperm of maize, wheat, barley, and rice all possess both classes. There have been efforts to characterize the differing specificities of branching enzymes, but much remains to be learned. Analysis of recombinant maize enzymes revealed that with amylose as the substrate, BEI transfers longer chains than BEIIb (Guan and Preiss, 1993). Substrate preference in vitro also varied, with BEI being more active toward amylose and BEIIb preferring amylopectin. Similar results were obtained with purified wheat BEs (Morell et al., 1997). Defining the specificity of individual BEs is complex and will involve consideration not only of the chain lengths transferred, but also definition of the reaction rates with respect to cleavage distance from a reducing end, a non-reducing end, or a branch linkage. Specificity may also be expected regarding proximity of the acceptor site, i.e. the newly formed branch, to these chemical reference points. There has been little comprehensive work to define these parameters.

Whether SSs and BEs can produce a glucan able to autoassemble into semicrystalline amylopectin is not yet known. Molecular cloning advances raise the possibility of reconstituting an amylopectin biosynthetic system with specific BE/SS combinations. In one relevant study, *Escherichia coli* was utilized to produce maize BEI and BEIIb in the same cells (Guan et al., 1995). The polymers produced in conjunction with the prokaryotic glucan synthase were distinct from amylopectin and resembled the relatively unordered molecule glycogen. The BE catalytic functions, therefore, are not likely to entirely determine the higher order structures of amylopectin. More sophisticated reconstituted systems, including multiple isoforms of SS and BE expressed at the appropriate levels, might produce glucan exhibiting some hierarchical structures of amylopectin, e.g. side chain clusters or blocklets. Such results would allow strong conclusions regarding which enzymes are required for synthesis of the amylopectin crystal. A complementary method employs mutations to identify factors needed for normal amylopectin synthesis. Genetic approaches alone cannot identify the specific function of an enzyme, although they do offer a powerful means of identifying factors that must be considered as possible direct determinants of amylopectin structure.

DBEs AND THEIR INVOLVEMENT IN AMYLOPECTIN BIOSYNTHESIS

DBEs catalyze the hydrolysis of $\alpha(1\rightarrow6)$ linkages (Fig. 3). Again, multiple isoforms are present, referred to here as isoamylase-type DBEs and pullulanase-type DBEs. Enzymatic and structural characteristics distinguish the isozymes (Nakamura, 1996; Beatty et al., 1999, and refs. therein). Isoamylase-type DBEs hydrolyze $\alpha(1\rightarrow6)$ linkages in denatured amylopectin, glycogen, and amylolytic derivatives (i.e. limit dextrins), but do not cleave branches in pullulan, a polymer of repeating $\alpha(1\rightarrow6)$-linked maltotriosyl units. Pullulanase-type DBEs (also called limit-dextrinases or R-enzymes) differ in that they readily attack pullulan and limit dextrins, but are less active or inactive toward glycogen and denatured amylopectin.

The biochemical nature of both DBE isoforms has been described recently. Higher plant isoamylase-type DBEs are multimeric, with molecular masses of approximately 340 to 500 kD for both the potato tuber and rice endosperm enzymes (Ishizaki et al., 1983; Fujita et al., 1999). This compares to the 83-kD monomer size generally conserved in plants, as shown by immunoblot analysis of crude cell lysates. Polypeptides of 95 and 83 kD were separated from the purified complex of potato, although these have not been further characterized. In the purified rice complex, only a single 83-kD band was detected by SDS-PAGE. Isoelectric focusing revealed two bands in the complex, although peptide mapping and sequencing showed that they result either from the same or from two very closely related polypeptides (Fujita et al., 1999). Activity was obtained from recombinant maize SU1 polypeptide purified from *E. coli*, which is consistent with a homomultimeric structure (Rahman et al., 1998). The possibility that some complexes may contain two or more related polypeptides cannot be excluded, however, considering that multiple forms are detected in non-denaturing zymograms, and that at least some species possess genes for two closely related isoamylase-type DBEs (see below). Pullulanase-type DBEs exist as monomers, although they share the interesting property with the isoamylase-type isoform that a single polypeptide apparently can exist in multiple forms that vary by pI (Nakamura et al., 1996a; Henker et al., 1998; Renz et al., 1998; Beatty et al., 1999, and refs. therein).

Amylopectin does not accumulate to normal levels when an isoamylase-type DBE is mutated, suggesting that this enzyme participates in starch biosynthesis. The maize mutation *su1−Ref* significantly alters starch biosynthesis in endosperm (Shannon and Garwood, 1984), and *Su1* codes for an isoamylase-type DBE (James et al., 1995; Rahman et al., 1998). The Arabidopsis gene *Dbe1* codes for an isoamylase-type DBE, and mutations therein prevent leaf starch from accumulating to normal levels (Zeeman et al., 1998). Mutation of the *Chlamydomonas reinhardtii* gene *STA7* affects an isoamylase-type DBE and also conditions a severe defect in storage starch biosynthesis (Mouille et al., 1996). Finally, the rice *Su1* gene codes for an isoamylase-type DBE, and mutations therein cause similar abnormalities in starch production (Nakamura et al., 1996b; Kubo et al., 1999). These parallel observations provide compelling evidence that interference with DBEs alters the process of amylopectin biosynthesis.

Conclusions from genetic observations are complicated by potential pleiotropic effects, i.e. one mutation may secondarily affect multiple enzymes. This is the situation in maize and rice, because both the isoamylase-type and the pullulanase-type isoforms are affected by *su1* mutations (Nakamura et al., 1996b; Beatty et al., 1999). In *C. reinhardtii* and Arabidopsis, however, the only observable defect is in the isoamylase-type DBE.

These results suggest that branch linkage hydrolysis is required for net amylopectin production. In all the of the above species, amylopectin content is significantly decreased by the mutations and the abnormal polysaccharide phytoglycogen (PG) accumulates (Shannon and Garwood, 1984). Branch frequency in PG is approximately 10%, about twice that in amylopectin. PG does not exhibit the higher order structures of amylopectin, presumably because the chain length distribution is weighted toward shorter linear segments, and long B chains with multiple branches are lacking. PG and amylopectin accumulate together in rice, maize, and Arabidopsis mutants (Nakamura et al., 1997; Zeeman et al., 1998). Despite its reduced concentration, the structure of the residual amylopectin in Arabidopsis leaves appears to be normal (Zeeman et al., 1998). In *C. reinhardtii sta7* mutants and rice plants carrying a specific *su1*− allele, there is a nearly complete loss of amylopectin (Mouille et al., 1996; Nakamura et al., 1997). PG is soluble and thus separate from amylopectin in granules. Both glucans reside in the same cells, however, as shown by microscopy of Arabidopsis leaves and maize endosperm (Shannon and Garwood, 1984; Zeeman et al., 1998). In rice there are regions of the endosperm that appear to lack granules or amylopectin, distinct from other areas containing amylopectin (Nakamura et al., 1997).

The above data indicate that if DBEs are not functioning properly, then glucosyl units normally destined for incorporation into amylopectin are diverted into a more highly branched molecule. To understand this phenomenon, it is first necessary to determine whether DBE deficiency is the causative agent of the biosynthetic alteration. The alternative view suggests that the affected DBE is needed for proper functioning of a different enzyme, and that this pleiotropic effect in turn causes amylopectin deficiency. If DBEs are needed for amylopectin biosynthesis, then a second question is whether they act directly along with SSs and Bes, or if they function in a distinct pathway that affects amylopectin biosynthesis indirectly. Because of the specific effects of the *C. reinhardtii sta7* and Arabidopsis dbe1 mutations, we take the viewpoint that PG accumulation is caused by the DBE deficiency, not from pleiotropic changes in another enzyme. This view is supported by the fact that all known PG-accumulating mutants lack at least one DBE, whereas if pleiotropic effects were responsible, then other types of mutations should be found to cause PG accumulation.

The hypothesis that DBEs function in amylopectin biosynthesis predicts that they are conserved in plants and located at the site of glucan formation. Both predictions are met by recent experimental evidence. Pullulanase-type DBEs are conserved in germinating seeds and leaves, indicating a degradative function. However, these enzymes also exist in starch-accumulating tissues during development, including endosperm of maize, rice, and barley, and pea embryos (Nakamura et al., 1996a; Zhu et al., 1998; Beatty et al., 1999; Burton et al., 1999). Intraplastidial locations are indicated in maize and pea by enrichment in the amyloplast fraction, and rice and barley by an amino terminal transit peptide. Active pullulanase-type DBEs were detected after cell lysis, indicating that they most likely have access to nascent amylopectin during its synthesis. Isoamylase-type DBEs also appear to be broadly conserved, again with plastidial locations in starch-accumulating tissues. This isoform has been identified in potato, maize, rice, *C. reinhardtii*, and pea (Beatty et al., 1999; Fujita et al., 1999, and refs. therein). Plastidial location of isoamylase-type DBEs during starch biosynthesis was demonstrated in maize endosperm (Yu et al., 1998) and pea embryos (Zhu et al., 1998).

Sequence comparisons suggest that isoamylase- and pullulanase-type DBEs diverged before establishment of the plant kingdom, and that each subfamily subsequently has been highly conserved. cDNAs coding for pullulanase-type DBEs are known from rice endosperm, spinach leaves, maize endosperm, and barley grain (Beatty et al., 1999; Burton et al., 1999, and refs. therein). Isoamylase-type DBE coding sequences are known in maize, rice, potato, barley, wheat, and two distinct Arabidopsis genes (James et al., 1995; Zeeman et al., 1998; Fujita et al., 1999; Sun et al., 1999). The same isoforms compared among different species are 60% to 80% identical. Between the two isoforms within a species, there is significantly less identity than that observed when either enzyme is compared with a prokaryotic pullulanase or isoamylase. Thus, isoamylase-type and pullulanase-type DBEs are likely to serve non-overlapping functions in glucan metabolism.

D-ENZYMES AND THEIR INVOLVEMENT IN AMYLOPECTIN BIOSYNTHESIS

Genetic observations also implicate D-enzyme as a potential factor in amylopectin biosynthesis. D-enzymes transfer a segment of one linear chain to another (Fig. 3). An internal $\alpha(1\rightarrow4)$ linkage is cleaved and the released reducing end is attached to a separate chain through a new $\alpha(1\rightarrow4)$ linkage. Mutation of the *C. reinhardtii* gene *STA11* causes loss of a D-enzyme without detectable effects on any other known starch biosynthetic enzymes (Colleoni et al., 1999a, 1999b). Less amylopectin accumulates when D-enzyme is missing, and its structure is altered by a significant increase in the frequency of short A chains. Linear maltooligosaccharides (MOS) accumulate abnormally in the soluble fraction from the mutants. In parallel to the arguments made for DBEs, D-enzymes may either modify an amylopectin precursor or participate in a separate pathway indirectly required for normal amylopectin biosynthesis.

The involvement of D-enzyme in amylopectin biosynthesis may vary depending on physiological conditions or tissue. The effects of *sta11* were observed only in conditions of nutrient starvation, not in exponential phase cells, and starved cells accumulated starch at much higher levels. Furthermore, no obvious changes in starch biosynthesis occurred in potato tubers bearing an antisense transgene targeting D-enzyme (Takaha et al., 1998). Thus, it remains to be determined if D-enzymes are generally involved in amylopectin biosynthesis, and in this regard it will be useful to seek mutations analogous to *sta11* in other species. Finally, the proposed involvement of D-enzyme in

amylopectin biosynthesis does not preclude a function in starch degradation.

D-enzymes are known from leaves of pea, spinach, and Arabidopsis, potato tubers, and *C. reinhardtii* (Colleoni et al., 1999). In several instances D-enzyme was found partially or entirely within the plastid fraction, and in potato the corresponding gene is expressed in developing tubers as starch is synthesized. To date, plant D-enzyme sequences are known only from potato (GenBank accession no. X68664) and an Arabidopsis gene (GenBank accession no. AC002409), and these two polypeptides are less than 30% identical.

MODELS PROPOSED TO EXPLAIN DBE FUNCTION IN AMYLOPECTIN BIOSYNTHESIS

In an early attempt to explain the phenotype of *su1*− maize mutants, PG was proposed to be a direct precursor of amylopectin (Erlander, 1958). The action of BEs on a linear glucan was suggested to produce PG, and partial debranching then would convert the precursor into mature amylopectin (Fig. 4A). This proposal is inconsistent with the facts that the M_r and chain lengths of PG are smaller than those of amylopectin, precluding the possibility that the larger glucan could be formed from the smaller by DBE activity alone.

Another proposal was based on identification in *su1*− maize of a novel BE called PG branching enzyme, which was able to convert amylose into PG (Manners, 1997). PG branching enzyme was reported to be absent from normal maize, explaining the presence of PG only in *su1*− mutants (Fig. 4B). In subsequent studies, however, similar BE activity peaks were purified from endosperm extracts of both normal and *su1*− mutant maize and rice (Boyer and Preiss, 1978; Nakamura et al., 1996b), so a BE unique to PG-accumulating mutants has not yet been specifically identified. Moreover, epistasis studies in maize revealed that PG accumulation is significantly reduced or absent in *ae*−*su1*− double mutants (Shannon and Garwood, 1984). Thus, BEIIb, the product of *Ae*, is largely responsible for PG synthesis, and this enzyme functions in non-mutant plants. Finally, direct demonstration that maize and rice *Su1* and Arabidopsis Dbe1 code for DBEs is difficult to reconcile with the production of a novel BE in the mutants. Despite these challenges, the possibility remains that BE activity in vivo is altered as a secondary effect of a DBE mutation.

The demonstration that maize *Su1* codes for an isoamylase-type DBE and that *C. reinhardtii sta7* mutants defective in this isoform lack starch completely prompted the modification of an earlier idea that PG is an intermediate in amylopectin biosynthesis (Ball et al., 1996). According to the glucan-trimming model, selected branches are removed from a precursor called pre-amylopectin, converting it to amylopectin (Fig. 4C). The substrate specificity of the maize isoamylase-type DBE suggests that in vivo it may cleave widely spaced branches more readily than those located closely together, thereby generating the asymmetrical distribution of branched and unbranched areas required for the formation of amylopectin side chain clusters. A discontinuous cycle of events involving elongation by SSs, branching by BEs, selective debranching by DBEs, and the formation of crystalline lamellae has been proposed. SS action on certain accessible non-reducing ends would then reinitiate the cycle.

A different model, referred to here as the water-soluble polysaccharide (WSP)-clearing model, was proposed recently to explain the fact that Arabidopsis dbe1 mutants simultaneously accumulate PG and starch granules with normal amylopectin in the same cells (Zeeman et al., 1998). This result is contrary to the glucan-trimming model, because in that view normal amylopectin would form without correct DBE activity. According to the WSP-clearing model, DBEs eliminate the products of a non-productive pathway in the soluble phase (Fig. 4D). SSs and BEs are proposed to work both in association with nascent granules and in the stroma, the latter pathway acting on MOS to produce small, branched glucans referred to here as WSPs. DBEs would function in the degradation of WSPs, thus preventing the diversion of SSs and BEs from their action at the granule surface. In this view, WSP synthesis competes with amylopectin formation, and DBEs would insure that the amylopectin pathway out-competes the non-productive diversion. Mutants lacking DBE would be less able to prevent glucan synthesis in the stroma, so that PG

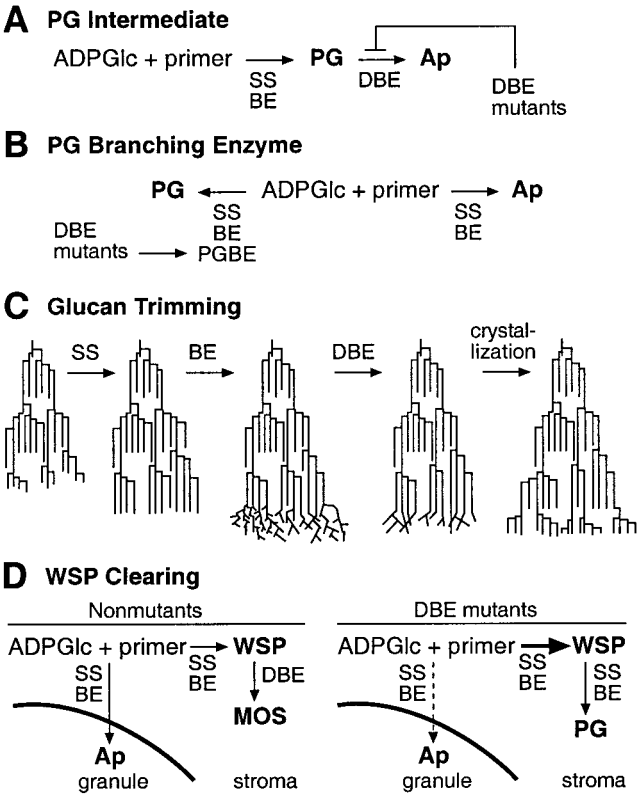

Figure 4. Models proposed to explain DBE function in amylopectin (Ap) biosynthesis. See text for details and references. The heavy arrow and the dotted arrow in D indicate the proposal that, in the absence of DBE, SSs and BEs spend proportionally more time converting WSP into PG and less time synthesizing amylopectin. The glucosyl units in MOS produced by DBE action during the elimination of WSP presumably are returned to the beginning of the pathway.

accumulates along with normal amylopectin and the yield of the latter is reduced.

GLUCAN TRIMMING REVISITED

We propose here some expanded aspects of the glucan-trimming model. The central point of this model is that transition of a precursor glucan from an unordered into an ordered state, i.e. crystallization, acts as a powerful organizing force and carbon sink driving the reactions of amylopectin synthesis. DBE deficiency is proposed to result in continued action of the SSs and BEs on a population of polysaccharides that fail to crystallize, thereby causing formation of both PG and amylopectin as end products of a diverging pathway. The model is depicted in Figure 5 and described as follows.

After the synthesis of nascent amylopectin molecules is initiated, they become substrates for SSs, BEs, and DBEs. Eventually, a lowest order structure suitable for crystallization is achieved, and then transition into a higher order structure occurs. Physical packing within crystallized regions is proposed to restrict access to biosynthetic enzymes. Once crystallization occurs, therefore, glucan is "fixed" into mature amylopectin. In this view, pre-amylopectin is defined as any branched glucan still accessible to the solute, whereas mature amylopectin is defined as the material that occurs after transition to the higher order structures. It is unclear how extensive the modification to pre-amylopectin must be to allow transition to mature amylopectin.

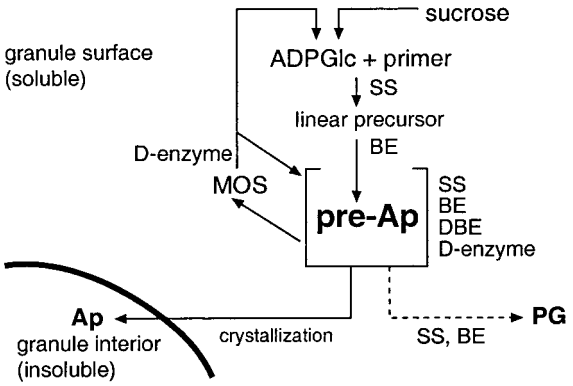

Figure 5. The glucan-trimming model involving amylopectin (Ap) crystallization as a driving force. The term pre-Ap indicates a heterogeneous population of branched glucans that are continually acted upon by the indicated enzymes. Thus, the precursor molecules exist as a broad spectrum of related structures. MOS is proposed to be released by DBE action. D-enzyme is proposed to transfer linear chains from MOS directly back into pre-amylopectin and also to participate in the generation of ADPGlc from MOS. Not indicated here is the possibility that MOS may serve as a precursor for SS. The possibility that D-enzyme also catalyzes the direct transfer of glucans among chains in pre-amylopectin is also indicated. Crystallization is proposed to remove pre-amylopectin from the aqueous phase and make it inaccessible to further enzymatic modification. The dotted line indicates that in the absence of DBE, pre-amylopectin can be continually acted upon by SSs and BEs to the point where crystallization is not feasible for physical reasons, eventually generating PG.

DBE function is proposed to be required for pre-amylopectin to attain a crystallization-competent structure, and this could not occur below a certain threshold level of DBE activity. In this condition, pre-amylopectin would remain accessible in the soluble phase to SSs, BEs, and other enzymes, eventually producing PG. The complete replacement of amylopectin with PG observed in *C. reinhardtii sta7* mutants might be explained by this mechanism. If DBE activity is reduced but not eliminated, then crystallization may proceed at a slower rate than normal because of the longer times needed to attain a structure competent for crystallization. Accordingly, pre-amylopectin on average would remain accessible to further enzymatic modification for an extended time. This continued action might convert the precursor into a form for which crystallization is no longer possible, fixing it into the PG diversion. Division between PG and amylopectin, therefore, would be a stochastic process depending on the rate and/or specificity of DBE activity, and both amylopectin and PG would form simultaneously. This could explain the simultaneous accumulation of both granular starch and PG in some mutants. DBE activity may be only partially reduced in these mutants because of the presence of additional isoforms of either the isoamylase or pullulanase type.

The glucan-trimming model must also explain why PG and amylopectin form in separate phases, i.e. the soluble fraction and starch granules, respectively. It is possible that the partitioning between amylopectin and PG occurs very early in the synthesis of any one molecule. Crystallization may occur initially at a very local level, perhaps as small as a single amylopectin side chain cluster. This initial event may provide an environment favorable for the capture of neighboring regions into the crystalline structure. If this initial seeding event is delayed by structural disorder resulting from excessive branching, then further disorder may accrue rapidly because of continued SS and BE action. Thus, crystallization-competent molecules may separate very early in their synthesis from those destined to be converted into PG. Another possibility is that on the surface of the granule, single pre-amylopectin molecules would exhibit both crystalline regions integrated into the granule and disordered regions in the soluble phase. These two areas might be separated by selective amylolytic activities or by physical shearing forces.

As a test of this model, it will be useful to characterize the glucans formed in plants that lack DBE function entirely. The construction of multiple mutants and/or transgenic approaches can be applied to construct such plants. If substantial amounts of normal amylopectin are formed, then it becomes unlikely that DBEs function to modify a direct precursor. Regarding the WSP-clearing model, observing that amylopectin is completely lost in DBE-null plants would mean that WSP synthesis is strongly favored over the normal productive pathway of amylopectin formation, and also that the return of glucosyl units from WSP into amylopectin synthesis is absolutely dependent on DBE function.

The characterization of additional mutations that cause PG accumulation may also serve to test the models. Recycling glucosyl units from WSP into amylopectin biosynthe-

sis may involve enzymes other than isoamylase-type DBEs, e.g. amylolytic enzymes involved in regenerating ADPGlc. Such functions might be identified genetically, which could provide support for the WSP-clearing model. Another potential means of testing the models is to characterize plants with alterations in DBE specificity but not its total activity, e.g. by introducing enzymes from other species or altering the relative levels of the native isoforms. The degradation of WSP might require less substrate specificity than the generation of crystallization-competent pre-amylopectin. Accordingly, comparing the effects of altered specificity with changed overall activity levels might provide support for one of these models.

POTENTIAL D-ENZYME FUNCTIONS IN AMYLOPECTIN BIOSYNTHESIS

The correlation between D-enzyme deficiency in *C. reinhardtii* and structural changes in amylopectin suggests a potential biosynthetic role for this enzyme. D-enzyme may play a direct role in amylopectin formation, or it could be involved in indirect recycling of glucosyl units from WSP or pre-amylopectin back into amylopectin biosynthesis. It is likely that DBEs function prior to the involvement of D-enzyme, providing MOS as the D-enzyme substrate. This is consistent with the role of DBEs as proposed in either the WSP-clearing model or the glucan-trimming model. Two possibilities have been proposed for the role of D-enzyme in amylopectin synthesis. First, D-enzyme may function as it does in prokaryotes for maltose utilization, such that the enzyme would convert MOS to Glc and other MOS chains (Boos and Shuman, 1998). The latter would be suitable substrates for phosphorylase and would thus be converted to Glc-1-P, and Glc could be phosphorylated by a hexose kinase. The glucosyl units would then be transferred to ADPGlc and reenter the biosynthetic process via SS.

This model could account for the decrease in total amylopectin biosynthesis that correlates with D-enzyme deficiency, although it does not explain the altered chain lengths observed in amylopectin from *C. reinhardtii sta11* mutants. A second possibility is that D-enzyme directly transfers glucan chains from MOS produced by DBEs into the A chains of amylopectin. This model could explain both the decrease in amylopectin biosynthesis and the altered chain length distribution. This idea is feasible because the transfer of glucosyl units from MOS directly into amylopectin has been demonstrated in vitro. An additional possibility is that D-enzyme uses donor and acceptor chains both covalently linked into amylopectin. Thus, D-enzyme should be considered in addition to SS and BE as a potential direct determinant of chain length distribution (Fig. 5).

CONCLUSIONS

The complex nature of starch biosynthesis was unexpected because of its simple composition and its apparently straightforward physiological function in carbon source storage. Comparing starch produced in plastids with glycogen in prokaryotes suggests that the biosynthetic pathway has changed in evolution because of selection pressure favoring a semicrystalline product. This property allows plants to store massive amounts of fixed carbon generated by photosynthesis. The change to amylopectin biosynthesis could have evolved through variation in the branching enzymes and glucan synthases previously involved in glycogen production. Alternatively, glycogen biosynthesis may have been modified to produce amylopectin through the adaptation of the functions of previously catabolic enzymes to modify glucan structure so that crystallization is promoted. The recruitment of catabolic enzymes might be a simpler explanation then evolving complex branching enzymes and glucan synthases capable of producing a crystallization-competent pre-amylopectin. This "capture" of catabolic enzymes into a biosynthetic pathway may be a common theme, because both D-enzyme and DBEs have been implicated in this regard. Continued genetic analysis is needed to determine whether other enzymes thought to have catabolic roles might also contribute to amylopectin biosynthesis. Most likely, however, the evolution of amylopectin biosynthesis occurred as the result of complex and interrelated alterations involving both SS and BE activity, as well as recruitment of DBEs and D-enzymes into some aspects of the process. These studies on a supposedly simple polymer such as amylopectin, which in fact demonstrates a high degree of hierarchical structure, are likely to be highly relevant to those trying to understand more elaborate hierarchical structures such as the plant cell wall or glycoproteins.

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