Progress in understanding the biosynthesis of amylose

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The storage of glucose in insoluble granules is a distinctive feature of plant cells. Biosynthesis of amylose, the minor low molecular mass fraction of starch occurs from ADP-glucose. This takes place within the polysaccharide matrix through the action of granule-bound starch synthase, the major protein associated with the granule. Recently, amylose has been successfully synthesized *in vitro* from purified granules. Two models have been proposed to explain the mechanism of amylose synthesis in plants. The first calls for priming of synthesis through small-size malto-oligosaccharides. The second suggests that glucans are extended by granule-bound starch synthase from a high molecular mass primer present within the granule. This extension is terminated through cleavage to produce amylose. This process is subsequently repeated to give several rounds of amylose synthesis.

Transient or long-term storage of glucose in starch granules can be considered as the last step in eukaryotic photosynthesis. Incorporation of glucose into these large structures (larger than the size of an individual bacterial cell) is increasingly being recognized as an exceedingly complex mechanism that is distinct from the glycogen-storing prochloron or cyanobacterial-like cells. The presence of only one type of sugar residue and only two distinct chemical linkages (α-1,4 and α-1,6) in starch granules had lead biologists to believe that the biosynthesis of amylose and amylopectin (the two distinct polysaccharide fractions found within starch) is a logical, economical and relatively simple pathway. These precepts were responsible for a general lack of interest in starch biosynthesis and restricted further analysis of the major polysaccharides. This situation has slowly changed and the emerging picture is that starch has a highly organized structure that requires the co-ordinated action of many different biosynthetic steps.

An overview of starch granule structure

Amylopectin, the major polysaccharide present in starch is one of the largest biomolecules known[1,2]. The complex organization of the α-1,6 branches within amylopectin is responsible for the semi-crystalline dense packing of glucans in insoluble granules (Fig. 1). Amylose, the minor fraction of plant starch granules, consists of smaller molecules with a low level of α-1,6 branches[2]. The comparative structures of amylose and amylopectin are summarized in Table 1. Because amylose does not adopt a semi-crystalline structure, and is thus not amenable to X-ray diffraction analysis, it is unclear how this polysaccharide interacts with amylopectin in the granule. Some structural biologists believe that long amylose-like chains are present in more or less regularly spaced amorphous cavities within the granules[3]. Others have proposed that this fraction can also infiltrate the amylopectin crystalline structure, because small-angle X-ray scattering experiments have shown that starches

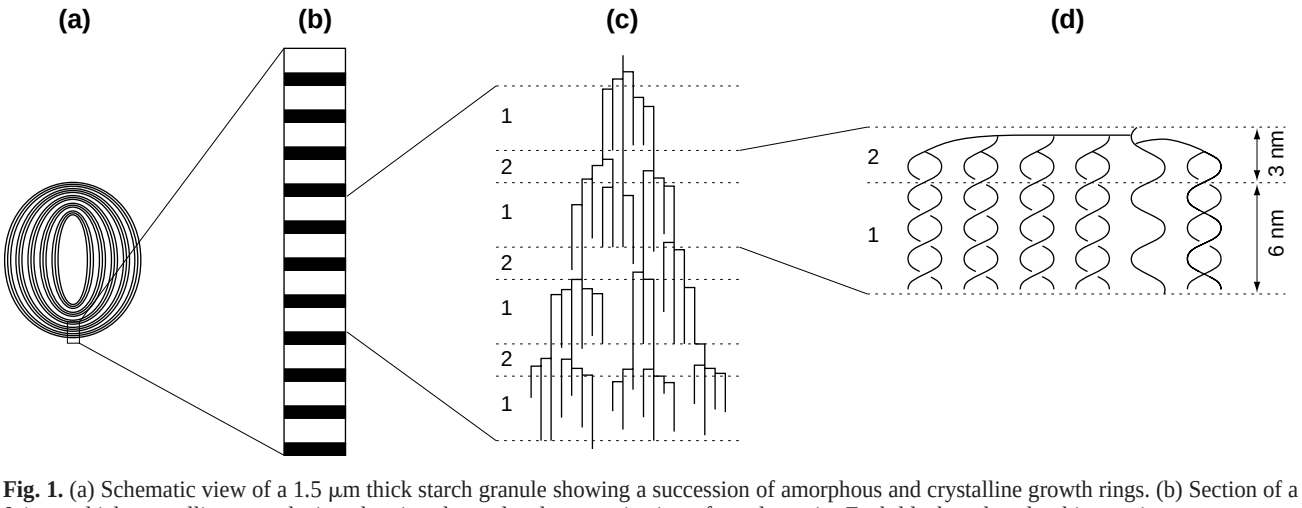

Fig. 1. (a) Schematic view of a 1.5 μm thick starch granule showing a succession of amorphous and crystalline growth rings. (b) Section of a 0.1 μm thick crystalline growth ring showing the molecular organization of amylopectin. Each black and each white section represents an amorphous and a crystalline lamella, respectively. Thus, the crystalline growth ring enlarged in (b) consists of a regular succession of 11 amorphous and crystalline lamellae. (c) Succession of seven lamellae in relation to the primary structure of a portion of an amylopectin molecule. Each line represents an α-1,4 linked glucan chain. The chains are interlinked by α-1,6 branches. The broken line delimits the sections appearing in the crystalline (1) and amorphous (2) lamellae. Note that most α-1,6 branches are included in the amorphous lamellae at the root of the chain clusters and that the glucans are pointing towards the granule's surface. (d) Part of the primary structure depicted in (c) is shown in relation to the secondary structure proposed for amylopectin. Each line represents an α-1,4 linked glucan chain. A single cluster is displayed. The parallel α-1,4 linked glucan double helices define the crystalline lamella. The base of the cluster contains most of the α-1,6 branches. The 6 nm size of the crystalline lamella corresponds to a length of 18 glucose residues.

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Table 1. The comparative structure of amylose and amylopectin

Characteristic	Amylose	Amylopectin
Weight percentage in the granule	15–35%	65–85%
Percentage of α-1,6 branches	<1%	4–6%
Molecular mass (Da)	10^4–10^5	10^7–10^8
Degree of polymerization	10^2–10^3	10^3–10^4
Chain-lengths	3–1000	3–50

with increasing amylose content display changes in the ratio of the amorphous to crystalline lamellae within the unit amylopectin cluster[4]. However, this particular study compared starches from different genotypes, some of which are known to differ in their amylopectin synthesis machinery: differences in amylopectin structure can lead to modifications in the X-ray diffraction pattern in the absence of amylose[5]. Therefore, it is not known whether the reported changes are due to differences in amylose content or because of the mutant amylopectin structures.

Applications of starches containing altered amounts of amylose

Over the past few decades, the importance of amylose-free (waxy) or high-amylopectin starches has been increasing. The *waxy* maize mutants first became popular as a substitute for cassava starch during World War Two. The development of amylose-free potato starch by genetic modification techniques[6,7], has led to a dramatic increase in interest in obtaining and using amylose-free starches. The great advantage of amylose-free potato starch is that the usual characteristics of potato starch, namely the low protein and fat content in combination with high molecular weight amylopectin, are retained, and combined with improved, environmentally friendly processing techniques for starch production. This type of starch dramatically reduces the need for postharvest chemical modifications, because amylose-free starch already has many of the characteristics (such as gel stability and clarity[8,9]) that are normally obtained only after chemical treatment.

An overview of the molecular and biochemical aspects of starch synthases

No mutants have been identified to date that selectively lack amylopectin, whereas mutations leading to the selective loss of amylose have been described in many species including waxy mutants of monocots (maize[10], rice[11], barley[12] and wheat[13]), amylose-free dicots (potato[14], pea[15] and amaranth[16]) and *Chlamydomonas reinhardtii*[17]. The groundwork in maize by Nelson and Rines[18], established that mutants that lack amylose have either simultaneously lost or modified their major granule-bound starch synthase activity. This correlates with the modification or disappearance of the major granule-associated protein which in most plant species ranges in size between 58–60 kDa. All these mutants build wild-type numbers of granules with a normal organization. It is, therefore, reasonable to assume that amylose synthesis occurs downstream of amylopectin. The synthesis of both amylopectin and amylose depends on the transfer of glucose in an α-1,4 position from ADP-glucose to the non-reducing end of growing chains. This elongation step is catalysed by multiple forms of ADP-glucose: 1,4-α-D-glucan 4-α-D-glucosyltransferases known as starch synthases. Starch synthases were initially discovered by de Fekete *et al.*[19] in association with starch granules. The major granule-bound activity was subsequently called granule-bound starch synthase (GBSSI). At

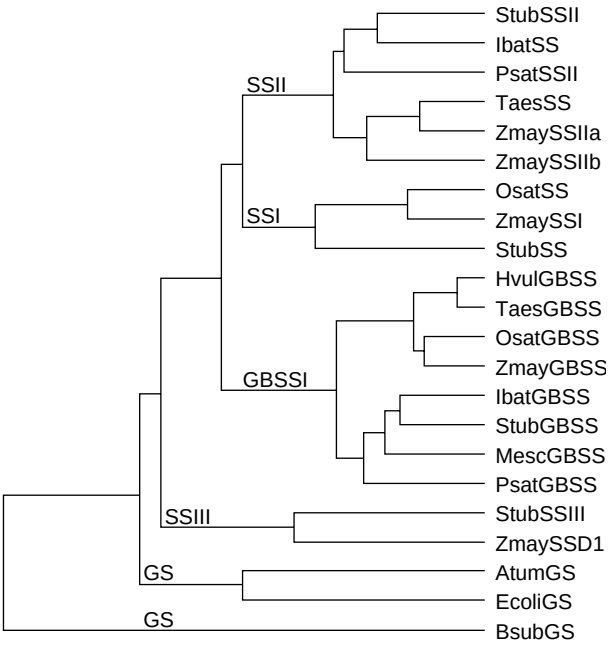

Fig. 2. Dendrogram showing the relationships between the different starch synthases and bacterial glycogen synthases. The amino acid sequences were aligned using the clustal method with PAM250 residue weight table. The identities of the proteins are: StubSSII, (*Solanum tuberosum* SSII)[29]; IbatSS (*Impomoea batatas* SS; C. Harn *et al.*, unpublished); PsatSSII (*Pisum sativum* SSII)[38]; TaesSS (*Triticum aestivum* SS; F. Walter *et al.*, unpublished); ZmaySSIIa (*Zea mays* SSIIa)[39]; ZmaySSIIb (*Zea mays* SSIIb)[39]; OsatSS (*Oryza sativa* SS)[40]; ZmaySSI (*Zea mays* SSI)[28]; StubSS (*Solanum tuberosum* SS; G.J.W. Abel *et al.*, unpublished); HvulGBSS (*Hordeum vulgare* GBSSI)[41]; TaesGBSS (*Triticum aestivum* GBSSI)[42]; OsatGBSS (*Oryza sativa* GBSSI)[43]; ZmayGBSS (*Zea mays* GBSSI)[44]; IbatGBSS (*Ipomoea batatas* GBSSI; Wang *et al.*, unpublished); StubGBSS (*Solanum tuberosum* GBSSI)[25]; MescGBSS (*Manihot esculenta* GBSSI)[45]; PsatGBSS (*Pisum sativum* GBSSI)[38]; StubSSIII (*Solanum tuberosum* SSIII)[46,47]; ZmaySSD1 (*Zea mays* SS dull1)[48]; AtumGS (*Agrobacterium tumefaciens* glycogen synthase)[24]; EcoliGS (*Escherichia coli* glycogen synthase)[22]; BsubGS (*Bacillus subtilis* glycogen synthase)[23].

first GBSSI was reported to use non-physiological concentrations of UDP-glucose[20], and ADP-glucose was subsequently discovered to be the preferred substrate[21]. Other forms of starch synthases exist either as soluble forms or as both soluble and minor granule-bound isoforms. A comparison of the available starch synthase sequences reveals that four distinct classes can be identified. The homology between and within these classes and with glycogen synthase from *Escherichia coli*[22], *Bacillus subtilis*[23] and *Agrobacterium tumefaciens*[24] is shown in Fig. 2. As expected, the genes from dicots are more closely related to each other than to the monocots, which are in turn more homologous to each other. The C-terminal region of the coding sequence of the four groups contains several conserved regions including the three short spans of amino acid domains I, II and III (Ref. 25), which are also present in glycogen synthase. Domain I contains the KXGGL consensus sequence which is believed to be the ADP-glucose binding site[26]. Domains II and III lie close to the C-terminus of the protein. The isoforms of starch synthase from the different classes share the highest homology between the region spanning KXGGL through to the C-terminus. In addition to this catalytic C-terminal domain, SSI, SSII and SSIII contain a highly diverged extension at the N-terminus, referred to as a 'flexible arm'[27,28]. This flexible arm is not present

in GBSSI. In addition, GBSSI appears to be exclusively granule-associated and was reported to lose most of its activity when dissociated from the the starch granule. Low amounts of soluble starch synthases were found in association with the granule[28–30]. GBSSI mutants in maize, pea and potato contain no amylose in spite of the fact that other isoforms of starch synthase are associated with the starch granule in all three organisms[28–30]. This indicates that granule binding capacity is not in itself sufficient for amylose production. The cloning of the GBSSI gene in potato allowed confirmation of the mutant analysis[6]. Potato plants expressing antisense constructs to GBSSI at different levels were instrumental in proving that amylose biosynthesis is occurring both within the starch granule and at the outer surface. This provided evidence that the reduction in amylose content was not as a result of a lower amylose content throughout the starch granule, but only a reduction in amylose synthesis in the outer parts of the granule. The GBSSI activity was confined to a core within the starch granule where the amylose was produced. The starch in this core, the size of which was dependent on the amount of GBSSI protein present, was indistinguishable from normal wild-type starch and contained the same ratio of amylose to amylopectin[7].

Enzymes involved in the starch synthesis pathway and their possible role in amylose biosynthesis

Introduction of the α-1,6 branch proceeds through cleavage of a pre-existing α-1,4 linked glucan and transfer of the cleaved glucan in an α-1,6 position. The corresponding 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferases belong to two distinct families of branching enzymes with different substrate and product specificities. The branching enzymes are also found among the minor proteins associated with the starch granule. It is reasonable to assume that these granule-bound branching enzymes are responsible for the low level of α-1,6 branches found in amylose. The fact that mutants lacking GBSSI also lack amylose does not imply that GBSSI is the sole determining activity for amylose biosynthesis. Because the Michaelis constant (K_m) of GBSSI for ADP-glucose is five- to tenfold higher than that of the soluble starch synthases, amylose synthesis is particularly sensitive to substantial decreases in the supply of the ADP-glucose substrate. ADP-glucose pyrophosphorylase, which catalyses the formation of ADP-glucose from glucose-1-P and ATP, which is a major rate-controlling step in starch biosynthesis, therefore exerts some control on the final amylose content within the granule. This is demonstrated by either the disappearance or reduction in amylose content in mutants for both ADP-glucose pyrophosphorylase and phosphoglucomutase in pea and *Chlamydomonas*[31,32]. Moreover, QTLs controlling amylose synthesis in maize were found to be linked to genes encoding the endosperm ADP-glucose pyrophosphorylase. However, no reduction in amylose content was reported in maize mutants with decreased ADP-glucose pyrophosphorylase, while inhibition of this activity in potato using an antisense approach did result in substantial decreases in amylose content. The structure of the starch in these low ADP-glucose-containing mutants resembles that of transient starch, a form of the polysaccharide that accumulates during the day in leaf cells and is degraded at night according to the physiological needs of the plant. Leaf starch is known to contain less amylose than storage starches of sink tissues[33]. This can also be traced to restrictions in the supply of substrate. Indeed, starch synthesis in chloroplasts of leaf cells compared with that of amyloplasts in sink tissues is somewhat more restricted because the chloroplast is often actively engaged in the export of photosynthate. Abundant amylose synthesis by GBSSI is thus a distinctive feature of starch-storing tissues.

Amylose synthesis *in vitro*

The first attempts to analyse polysaccharide biosynthesis under *in vitro* conditions using purified starch granules were performed by the Nobel prize winner, Leloir[20]. In these studies, non-physiological concentrations of radiolabelled UDP-glucose (from 100 to 200 mM) were used to incubate native starch granules. Leloir *et al.*[20] concluded that both amylopectin and amylose incorporated equivalent amounts of label but that the amylose-specific radioactivity was threefold higher. It was also found that the enzyme readily added a few glucose residues to small malto-oligosaccharides (maltose and maltotriose), thus yielding soluble products with small extensions[20]. However, under these experimental conditions, this appeared to occur at the expense of polysaccharide synthesis in the granules. Leloir correctly interpreted these results as reflecting competition between the added malto-oligosaccharides and the native primer, which he considered to be present within the granules. At that time, starch granules were used as the source of enzyme to show that, by analogy with mammalian glycogen synthesis, nucleotide-sugars were indeed used as substrate for plant polysaccharide synthesis. However, Leloir *et al.*[20] did not realize that they were specifically assaying GBSSI, as it was not known that the enzyme was devoted to amylose synthesis. Using the same system, Recondo and Leloir[21] subsequently demonstrated that ADP-glucose was the preferred glycosyl-nucleotide for incorporation into starch, and that the enzyme worked faster at lower, more physiological concentrations of ADP-glucose. This pioneering work was re-examined 25 years later during an investigation into polysaccharide synthesis by starch granules of sweet potato[34]. Baba *et al.*[34] were, by then, aware that the enzyme responsible was GBSSI and that their results were to be interpreted in the context of amylose synthesis. The system was improved by separating amylose and amylopectin more accurately and by investigating the structure of the products synthesized *in vitro*. It was found that radioactivity was incorporated into amylose and amylopectin[34]. However, although amylose remained the predominantly labelled species, they showed that the newly synthesized material included unusually long external amylopectin chains. None of the intermediate-sized chains, which constitute the semi-crystalline clusters of standard amylopectin, was labelled under these conditions. The authors therefore questioned the genetic evidence that GBSSI was solely responsible for amylose synthesis because, *in vitro* at least, the enzyme was engaged in some aspects of amylopectin synthesis. These *in vitro* results appeared at first to be confirmed by *in vivo* studies in *Chlamydomonas reinhardtii*[35]. In specific mutant backgrounds, granule-bound starch synthase controlled the amount and structure of the amylopectin synthesised. Once again the differences were seen solely in the long chains of the amylopectin fractions. However, neither group realized that these amylopectin molecules were, in fact, intermediates in normal amylose synthesis.

Although malto-oligosaccharide-prompted synthesis was ignored in these *in vitro* experiments[34], Denyer *et al.*[36] reported experimental conditions where label was selectively found in amylose when starch granules from pea leaves were incubated in the presence of both radiolabelled ADP-glucose and maltotriose. In the absence of maltotriose, all the label was detected in amylopectin, suggesting that GBSSI-catalysed amylose synthesis requires the presence of small malto-oligosaccharides[36]. One mechanism by which malto-oligosaccharides could trigger amylose synthesis, which is in agreement with earlier observations[20], is if they are used as primers by GBSSI (Fig. 3). In their absence, GBSSI would participate as a minor component of the amylopectin synthesis machinery. The maximal size of the soluble malto-oligosaccharide that can reach GBSSI will be conditioned by the porosity of the starch.

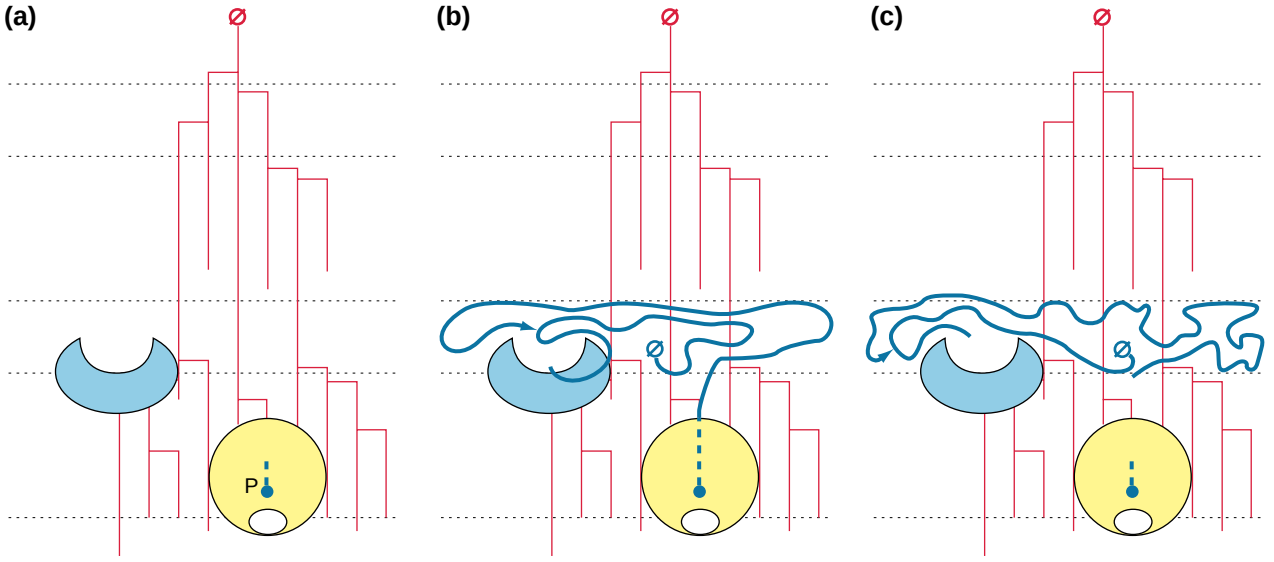

Fig. 3. Malto-oligosaccharide primed synthesis of amylose by granule-bound starch synthase I (GBSSI). All reaction steps occur within the very dense polysaccharide matrix within the granule. The amylopectin matrix is shown in red. A blue broken line symbolizes the presence of the growing glucan within GBSSI (yellow), and an unbroken blue line represents the amylose outside of the enzyme. The symbol (•) denotes a non-reducing end whereas (∅) symbolizes a reducing end. A branching enzyme is shown as a blue crescent-shaped structure. The polymerase activity at the catalytic site of GBSSI is shown as (P). Black broken lines indicate the presence of amorphous and crystalline lamellae as in Fig. 1. (a–c) Three different times during the first round of amylose synthesis by GBSSI. (a) A malto-oligosaccharide has diffused through the polysaccharide matrix and reached the GBSSI catalytic site. (b) The immobilized enzyme has elongated the chain processively, thus pushing the chain inside the starch granule. A random collision with a branching enzyme in the path of the growing amylose chain results in branching of the molecule towards the reducing-end. (c) Finally, a novel malto-oligosaccharide has reached the catalytic site, resulting in release of the amylose molecule and priming of a novel chain.

The basic difference between these recent results[36] and those obtained earlier[20,34] is the extent to which amylose synthesis occurs in the absence of malto-oligosaccharides. In the earlier studies, starch granules were incubated from 1 to 3 hours[20] and 24 hours[34], respectively. In the later study[36], the incubation was restricted to 1 hour. In *C. reinhardtii*, significant amylose synthesis occurred one hour after the addition of maltotriose[37]. To resolve these apparent contradictions, the kinetics of the reaction was followed over a time course of 1–24 h in the absence of malto-oligosaccharides[37]. Although no synthesis of amylose occurred during the first hour, this did occur at later stages, but no trace of malto-oligosaccharides was found in the incubation media. After 24 hours of *in vitro* synthesis, the newly synthesized amylose was subjected to detailed structural analysis and was found to be identical to the native amylose present in starch granules. By carefully monitoring the radioactivity in each fraction and in the whole starch granule, it was apparent that the amylose synthesis rates went on to exceed net polysaccharide synthesis within starch[37]. This could be explained only if amylose was synthesized at the expense of the long chains built by GBSSI on amylopectin. Moreover, the onset of amylose synthesis correlated with the end of the extension stage for the long amylopectin outer chains. Also, long-chain amylose synthesis preceded short-chain amylose biosynthesis. This led to the hypothesis that GBSSI was continuously using amylopectin as a primer and extending a long outer chain. When the extended molecule had reached a sufficient size, an endo-type of cleavage event would terminate the mature amylose molecule. To test their hypothesis, the authors performed pulse–chase experiments on purified *Chlamydomonas* starch granules[37]. After feeding short (30 min) pulses of radioactive ADP-glucose, the label, which at first was confined to the amylopectin molecule, was slowly chased into the amylose fraction. After a 24-hour chase, over 60% of the label was found in the amylose fraction. Once again, high mass amylose appeared first, suggesting that once cleaved the amylose chains were not further used as substrate by GBSSI. Two distinct mechanisms of chain cleavage were postulated (Fig. 4). In the first, amylose cleavage occurs downstream by a starch hydrolase within the granule (Fig. 4 panel I). To achieve several rounds of amylose synthesis upon an amylopectin primer, GBSSI would have to extend another molecule from a novel accessible non-reducing end, thereby implying some freedom of movement. In the second mechanism (Fig. 4 panel II), GBSSI is postulated to be directly responsible for cleavage of the amylose chain. The hydrolytic reaction, in this case, would be triggered by steric hindrance encountered during the progress of the growing amylose chain. The presence of amylose in the pre-existing amylopectin semi-crystalline matrix is suggested by X-ray diffraction analysis of granules subjected to *in vitro* synthesis of amylose. Preliminary results suggest that amylose pushes amylopectin from the A- into the B-type of crystalline lattice[37]. Indeed the B-type allows room for infiltration of one or two amylose chains in the middle of the amylopectin crystal.

Future perspectives

The synthesis of amylose *in vitro* by GBSSI from a high mass primer present within the granule calls into question the mechanism of malto-oligosaccharide priming that has been proposed[36]. Whether malto-oligosaccharide primed synthesis occurs at all *in vivo* will require analysis of the respective malto-oligosaccharide and ADP-glucose concentrations. Moreover, a detailed structural analysis of the composition of this malto-oligosaccharide fraction at the time of amylose synthesis is essential. It is possible that some of these components will not be available for amylose priming because of their size and the restricted porosity of the starch granule. Malto-oligosaccharide priming of amylose synthesis predicts a correlation between their concentration within the plastid and the rate of amylose synthesis. This correlation remains to be established in backgrounds with altered malto-oligosaccharide concentrations.

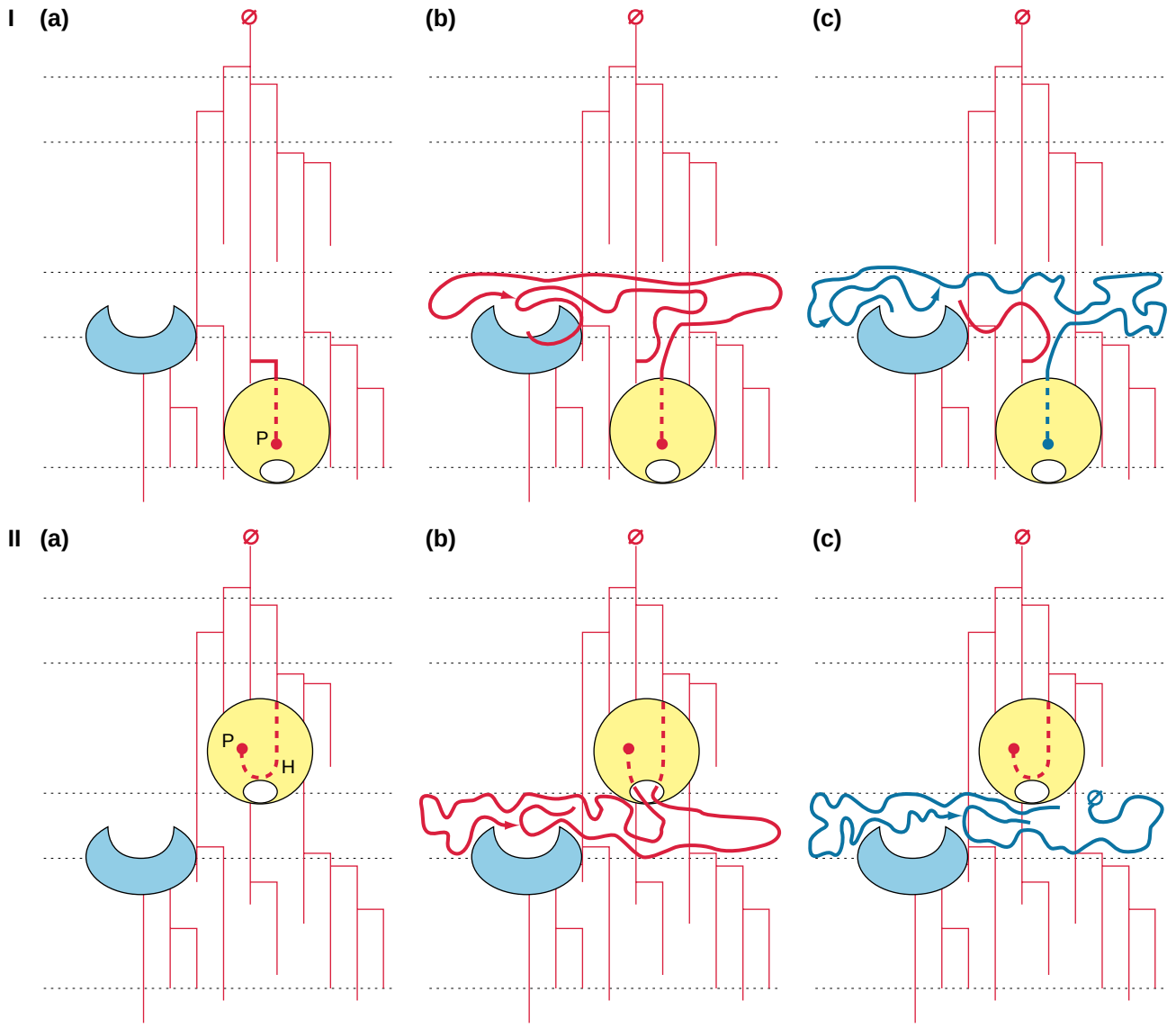

Fig. 4. Possible models explaining amylopectin primed amylose synthesis by immobilized granule-bound starch synthase I (GBSSI). All reaction steps occur within the very dense polysaccharide matrix within the granule. The amylopectin matrix is shown in red and the amylose in blue (lines). The symbol (•) denotes a non-reducing end, whereas (∅) symbolizes a reducing end. A branching enzyme (BE) is shown as a blue crescent-shaped structure. The polymerase activity at the catalytic site of GBSSI (yellow) is shown as (P) (panels I and II) and (H) indicates a postulated hydrolase activity (panel II). In panel I we show a BE-mediated cleavage: cleavage by a hydrolytic enzyme is also possible but is not shown. In panel II hydrolysis at site H within GBSSI occurs near to the site of polymerization, ensuring that the non-reducing end is close to the site of synthesis for reinitiating the next round of amylose biosynthesis. This is in contrast to panel I, where the next round of biosynthesis is dependent on the availability of a new non-reducing end. Black broken lines indicate the presence of amorphous and crystalline lamellae as in Fig. 1. (a–c) Three different time stages during the first round of amylose synthesis by GBSSI. In contrast to Fig. 3, the primer used in panel I (a) and (b) is the amylopectin itself. The non-reducing end displayed in panel II (c) is a new one generated through GBSSI-mediated cleavage.

It appears likely that a high mass polysaccharide is being used by GBSSI in the granule to extend amylose chains. Whether this primer represents standard amylopectin or a specific subset of amylopectin-like structures remains to be determined. It appears that GBSSI requires an organized granule to become fully active because *in vitro* studies have highlighted the very low specific activity of the unbound recombinant enzyme (C. Martin, pers. commun.).

Another important aspect for future research will be to ascertain the mobility of GBSSI within the polysaccharide matrix. An immobile enzyme would require the presence of built-in hydrolase activity in the enzyme itself. The ability to synthesize amylose *in vitro*, the availability of many point mutations of GBSSI, and our improved understanding of the function of GBSSI at the molecular level, give us confidence that a more thorough understanding of amylose synthesis is in sight. This knowledge could be usefully employed by those interested in starch biosynthesis and in plant breeding programmes. It will also be of relevance to work on enzymes in a variety of unusual physical environments similar to that provided by the polysaccharide matrix.

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