Structure, function and regulation of the enzymes in the starch biosynthetic pathway.

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1. Statement of the Research Problem.

The biosynthetic enzymes of starch and glycogen.

Starch, after cellulose, is the most abundant polysaccharide in nature and is the major reserve polysaccharide in green plants. It is the primary source of caloric intake in humans and is becoming increasingly important as a renewable industrial biomaterial. The amount of starch harvested worldwide exceeds 10^9 tons per year. Around 2 x 10^7 tons of starch are used exclusively in industrial applications and this number is expected to rise as renewable sources replace petroleum-based sources of various materials.[1] Significant interest is now focused on strategies for altering both the quantity and properties of starch from various crops. For example, there is acute interest in producing starches with high amylose content because of its resistantance to digestion. Such resistant starches have been shown to have positive health effects and are thought to contribute to the advantages of a high fiber diet.[2] Modification of the starch biosynthetic enzymes provides a proven and fertile avenue for the achievement of these goals.[3] Starch is a polymeric material consisting of α-1,4-linked glucose units and α-1,6 linkages that serve as branch points for the polymer. Starch granules contain two types of glucose polymers, amylose which consists almost exclusively of linear α-1,4-linked polymers, and amylopectin which consists of highly branched polymers. The formation of a mature starch granule occurs in the plastid and is a complex process, involving many different enzymes including amylases, isoamylases, pullulanases and phosphorylases, that tailor the developing granule and are also important for starch mobilization. The starch granule has a characteristic structure made up of alternating crystalline and semi-crystalline zones.[1] Though the biochemical pathway for starch production is identical in all plants, as are most of the enzymatic activities associated with it, the starch produced is unique to each plant species. Even within the same plant species, distinct starch types are produced in different organs. It is clear from a number of studies that these differences are largely due to the relative activities and specificities of the starch biosynthetic enzymes.[1]

Our overarching goal is to begin to delineate in molecular detail the structure, mechanisms, specificity and regulation of the enzymes that make up the pathway for starch and glycogen biosynthesis in plants and bacteria, respectively. This knowledge will enable the rational redesign of these enzymes to both increase starch production and alter the properties of starch.

2. Specific objectives.

2.1 Glycogen Synthase (GS).

Our structures of both the open and closed forms of *E. coli* GS (EcGS) bound to a variety of ligands have shed insight into the basic mechanism of the enzyme. Our structure of EcGS bound to maltodextrins have identified potentially important maltodextrin binding sites on the surface of the enzyme that were previously completely unknown. It was, in fact not known that GS binds glucans on its surface outside of its active site. These studies naturally lead to many questions regarding the function of the enzyme that we will now pursue:

2.1.1. We will mutate the residues that interact with oligosaccharides outside the active site of GS and assay these mutants to understand the importance of these binding sites for enzyme activity.

2.1.2. We will crystallize EcGS bound maltooctaose and maltododecaose (M8 and M12) to determine how the external glucan binding sites might work together with each other or with the active site in the function of the enzyme.

2.1.3. We will expand our efforts to determine the structure of a bonafide Starch Synthase. We will investigate a number of organisms in this effort and will use a variety of techniques to produce a crystallizable protein construct. We will first focus on granule-bound starch synthase from potato, since we have already overexpressed and purified significant quantities of this protein, and we will extend our efforts to a number of different enzymes.

2.2. Branching enzyme (BE) structure, specificity and mechanism.

Our structure of a truncated form of *E. coli* BE has served to orient our thinking regarding the structure and function of the enzyme.[7] Though the active sites of all the enzymes in this family are similar, indicating a mechanistically similar reaction for all, what distinguishes these enzymes remains an open question. It is also not clear what gives rise to the differences in substrate and product specificity exhibited by the different BEs. An understanding of how this enzyme binds to larger glucan chains will be critical to answering all of these basic questions. Our recent structures of BE bound to a variety of oligosaccharides including cyclodextrins, maltohexaose and maltoheptaose (M6 and M7) have shown that the enzyme's active site may not strongly bind the substrate, but binding is instead dependent on binding sites relatively far from the active site. We will probe this hypothesis in a number of ways:

2.2.1. We will continue to mutate all of the oligosaccharide binding sites identified in our crystallization experiments and assay them both for activity and chain transfer specificity to determine their importance for the activity and specificity of the enzyme.

2.2.2 The best way to test the hypothesis that glucans may bridge the active site and the external binding sites identified in our structures is to produce a structure of the enzyme bound to a glucan of sufficient length to bridge the external binding sites and the active site. We now have such substrates in homogeneous form thanks to Professor Hwa Park, University of Enchon in Korea, a world expert in enzymatically derived sugar polymers. We also now have maltododecaose (M12). This glucan is far longer than either the shortest glucan, hexaose (M6) or the glucan most favored for transfer (8-10 units). It should therefore provide an effective model for the bonafide substrate in the branch transfer reaction.

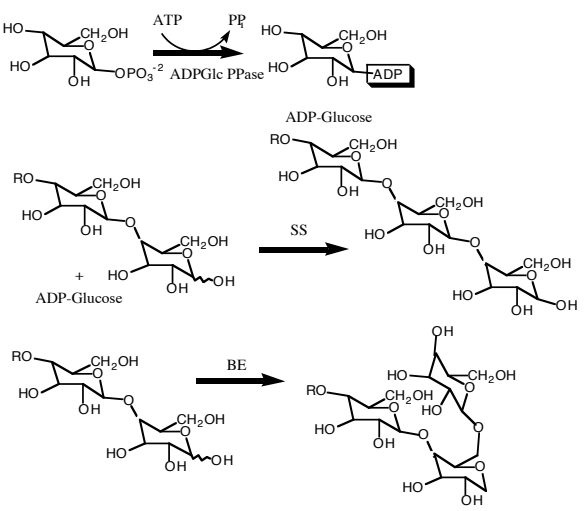

Figure 1. The bacterial glycogen and plant starch biosynthetic pathway.

2.2.3. Some of the glucan binding sites identified in our *E. coli* BE structures appear to be at least partially conserved in the plant BEs. We will explore the role of these potential glucan binding sites by making a number of mutants in these sites. We will make these mutations in examples of all of the isozymes found in plants, BEI, BEII, BEIIa and BEIIb, as the various isozymes play distinct roles in starch granule synthesis.[8] We will initially focus on the enzymes from rice and maize, as we have already begun cloning, overexpressing and purifying these enzymes to homogeneity. In addition, these enzymes are the best characterized of the plant BE isozymes.

2.2.4. We will continue in our efforts to crystallize and determine the structures of the isoforms of the plant branching enzymes bound to glucans. This work is critical as many of the glucan binding sites identified in our structures of the *E. coli* enzyme do not appear to be conserved in the plant enzymes. In addition, there is an N-terminal domain found in plant BE II isozymes whose structure is still unknown. We will first focus on determining the structure of BE I from rice bound to glucans as the structure of the apo form of this enzyme has already been determined.[9] We will also focus on producing crystals and determining the glucan-bound structures of both BE IIa and BE IIb from this organism. Though highly homologous, the differences in the roles these enzymes play *in vivo* and the differences in their substrate preferences, activity, and branch chain specificity indicate that they recognize glucan polymers in distinctly different ways.

3. Background.

The glycogen (in bacteria) or starch (in plants) biosynthetic pathway consists of the synthesis of ADP-glucose from glucose-1-phosphate and ATP catalyzed by ADP-glucose pyrophosphorylase (ADPGlc PPase); polymerization of glucose via α-1,4 linkages using ADP-glucose as a building block catalyzed by glycogen or starch synthase (GS or SS); and finally branching of these polymeric chains via α-1,6 linkages

catalyzed by starch branching enzyme (BE) (**Figure 1**). Numerous studies have shown that the quantity and properties of the resulting starch granule are largely dependent on the activity and specificity of the three major enzymes of the pathway and their isoforms.[1, 3b, 10] Therefore modification of the enzymes directly involved in granule synthesis will be required to tailor the properties and quantities of starch in an individual plant. A rational approach will require a detailed molecular understanding of both the mechanism and determinants of specificity for each of these enzymes. Atomic resolution structures of these enzymes are essential but not sufficient to achieve this goal. Additional structures of enzyme/substrate, enzyme/inhibitor and enzyme/effector complexes will be key to understanding the mechanisms and specificities of the enzymes, while mutagenesis and biochemical assays will be required to confirm the hypotheses generated by the structural data. Our work has been instrumental in initiating these studies as we are responsible for the first structures of two of the three enzymes in the pathway[7, 11] and published the first structure of a glycogen or starch synthase, in its catalytically competent conformation. We also showed, for the first time how a starch biosynthetic enzyme (EcBE) might recognize its polymeric substrate.[12] Our primary focus in this grant period is to understand as much as possible the process by which glycogen synthases, starch synthases and branching enzymes interact with their complex polymeric substrates to confer specificity and optimize or modulate activity because it is this interaction that is clearly crucial in the determination of their function.

3.1. Starch and Glycogen Synthases.

Glycogen and starch synthase (GS and SS, respectively) are members of the large glycosyl transferase superfamily of enzymes containing at least 90 families and more than 7200 sequences. In spite of this diversity, and in contrast to the glycosyl hydrolase superfamily, a comparatively small number of folds have been identified [13] The overwhelming majority of families share only two basic folds, the GT-A and GT-B folds. GS and SS enzymes have a GT-B fold. In the GT-B fold the two domains are separated into two distinct protein domains. Glycosyl transferases can also be divided into two groups based on the stereochemical result of the reaction, which can be either inversion or retention or configuration. GT-A and GT-B fold enzymes are both capable of catalyzing either stereochemistry, but the mechanisms are obviously distinct.[13b] While the mechanism involving inversion of configuration is relatively well understood in these enzymes, the mechanism resulting in retention of configuration is less understood, especially regarding the GT-B fold enzymes.

GS and SS enzymes are members of the GT-B fold GT-5 family and catalyze transfers resulting in retention of configuration. Several structures of GT-B fold retaining enzymes have been determined including maltodextrin phosphorylase (MalP), trehalose-6-phosphate synthase (OtsA), Glycogen Phosphorylase (GP), α-1,3 glucosyltransferase in lipopolysaccharide core biosynthesis (WaaG), and T4 bacteriophage α-glucosyltransferase (AGT) and all have shown striking similarities in the structure of their active sites, indicating a similar mechanism for all.[13b] In addition to the NDP-sugar dependent glycosyl transferases the glycogen phosphorylases (GP and MalP) also have similar active site structures, though in these enzymes the nucleotide diphosphate moiety is replaced by PLP and the substrate inorganic phosphate.

Glycogen or starch synthase (GS or SS) is responsible for glucose chain elongation. Four isoforms of SS have been identified (Granule-Bound Starch synthase (GBSS), SSI, SSII and SSIII) with many plants expressing all four of these isoforms. While GBSS is bound to the starch granule and is exclusively responsible for the formation of amylose, SSI, SSII, SSIII, BE and other debranching enzymes are involved in the production of amylopectin. Studies in starch granules have shown that GBSSI exhibits processivity, synthesizing long glucan chains from short-chain substrates, while SSII is nonprocessive, rarely adding more than one glucose unit at a time.[14] *In vitro* experiments using enzymes expressed in bacteria have shown that potato GBSSI has approximately ten-fold lower activity for elongation of maltotriose relative to potato SSII, with neither enzyme exhibiting processivity[15]. However, GBSSI is both allosterically activated more than ten-fold and becomes processive in solution when bound to amylopectin. SSII displays neither of these properties.

While no structures of an SS have been published, structures of GS from four organisms are known, *Agrobacterium tumefaciens* (AtGS),[16] the thermophilic organism *Pyrococcus Abyssi* (PaGS),[17] our structures of the *E. coli* in both its resting state and active conformation,[18] the structures of yeast GS (yGS) in both its resting and allosterically active states and most recently the structure of PaGS bound to oligonucleotides in its closed conformation.[19]

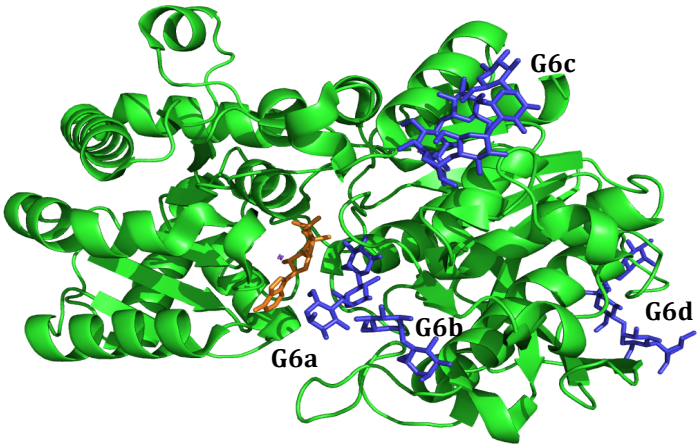

Figure 2. Structure of EcGS bound to M6. EcGS, green ribbon, M6, blue, ADP, orange .

In the last grant period, we completed and published a series of structures of *E. coli* Glycogen synthase (ECGS) in both the resting "open" conformation and the "closed" catalytically competent conformation (**Figure 2**). The structure of the open conformation confirms that the large domain motion appears to be a characteristic of all GS and probably SS enzymes as well, making them distinct from the GT-B fold retaining glycosyl transferase structures whose structures are known. An overlay of the "open" and "closed" structures of ECGS shows the more than 9 Å domain motion required to convert the "open" structure to the catalytically active "closed" conformation.[18] This essentially represents a motion of the entire domain to produce a closed active site. Our structures of EcGS bound to ADP and glucose gave important insights into the mechanism of the enzyme and elaborated the roles played by most of the residues in the active site. The recent structures of allosterically activated yGS and PaGS in their closed conformation served to confirm most of the results gleaned from our structures. Active site residues were in essentially identical positions to those of EcGS in both of these enzymes, and likely play the same roles as those in EcGS, though there is yet to be a structure of any other GS with a substrate bound in the active site.[19a, 19c, d] The yGS structure is bound by Glucose-6-phosphate, which is also the allosteric activator for the enzyme. Allostery is controlled by a rearrangement of the tetramer that leads to domain closure. Thus a similar motion seen in the resting state and activated state structures of EcGS is allosterically controlled in yGS.[19c, d]

We also determined the structure of the catalytically inactive E377A mutant EcGS bound to M6 (**Figure 2**).[12] As expected, M6 was bound near the active site, with its first glucose well positioned to accept the glucose from ADPGlc.[18] In addition to the M6 bound in the active site, three other M6's were also found to interact with EcGS, all to the N-terminal domain (**Figure 2**). These binding sites reside far from the active site, between 16 Å and 19 Å from the G6b and G6d binding sites, while the G6c binding site resides almost 30 Å from the active site, on the opposite side of the molecule. At present the importance of these active sites is not clear, but we have begun, and will continue a program to discover, the importance of these sites in EcGS. Though speculated about for a number of years, this is the first time that such external glucan binding sites were identified on an enzyme in the starch biosynthetic pathway.

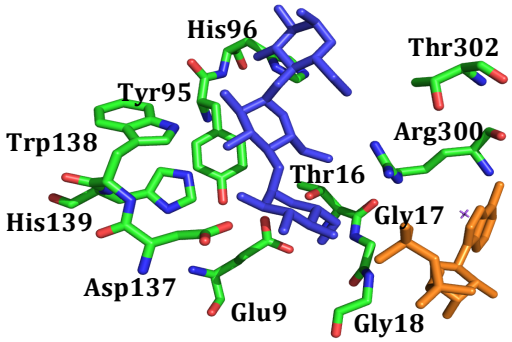

Figure 3. Residues that interact with M3 in the G6a EcGS site. ADP, orange, M3, blue, residues colored by atom.

More recently, the structures of maltodextrin complexes were determined for both yGS and PaGS. Surprisingly, neither structure evidenced any binding of maltodextrin in the active site, as seen in the EcGS/M6 structure. Interestingly, though the channel occupied by M6 in EcGS is strongly conserved in many bacteria and is essentially identical in GBSS enzymes,[12] it is significantly different in both PaGS and

yGS, indicating that they recognize glucan differently than EcGS and the plant enzymes do. Nevertheless, it is unclear as to why these enzymes did not bind glucan at or near their active sites. On the other hand, both enzymes bound maltodextrins on their surfaces, tens of angstroms from their active sites, as was seen for EcGS. Though few of the interacting residues were conserved, PaGS bound a maltodextrin in a location almost identical to the EcGS G6d site.[19a] Mutation of this site had a substantial deleterious effect on the activity of PaGS for glycogen, but little effect when M6 is used as substrate. Mutation of a Tyr (Tyr239) in a similar location in Human GS caused similar biochemical effects (WT with M6, much slower with glycogen), abrogated human GSs localization to glycogen and decreased production of glycogen *in vivo*. These results clearly show that the binding site we identified in EcGS is functionally significant in distantly related GSs. Human GS is far less homologous to EcGS than are the plant enzymes (less than 12% identity for human GS versus over 30% identity for most SSs), (alignment using DNASTAR, gap penalty 10, gap length penalty 0.2).

3.2. Branching Enzyme. Branching Enzyme (BE) is found in all organisms that make either starch or glycogen. It is a member of the glycosyl hydrolase superfamily of enzymes which includes over 80 families, and within this family belongs to the GH-13 "α-amylase" family of enzymes.[13a, 20] These include the α-amylases, isoamylases, cyclodextrin glucantotransferases, pullulanases, the debranching enzymes and others. All of these enzymes act on α–linked glucans, catalyzing either hydrolysis or glycosyl transferase reactions resulting in retention of configuration at the anomeric carbon. All share a common TIM-barrel fold consisting of an 8 β-sheet barrel surrounded by 8 α–helices. In addition to the central fold containing the catalytic domain, our structure of *E. coli* BE (EcBE) showed that EcBE also has a Greek key β-barrel C-terminal domain found in α-amylase and a carbohydrate binding module 48 (CBM48) domain found N-terminal to the central, catalytic domain (**Figure 4**).[2, 4, 21] N-terminal to the CBM48 domain is another β-barrel domain structurally homologous to the CBM48 domain. This domain was first seen in the structure of the BE from *Mycobacterium tuberculosis* (MtBE) as this domain was cleaved off of EcBE for crystallization. Removal of this domain had only modest effects on overall activity, but it had significant effects on the transfer chain specificity of EcBE. While the full length protein predominantly transfers shorter chains of between 7-15 glucose units in length, removal of the N-terminal domain altered the transfer-chain preference to longer chains containing 15-25 glucose units. Substantial amounts of chains as long as 30-40 residues were transferred with this mutant, indicating that the far N-terminus of *E. coli* BE has a significant effect on branch chain specificity.[22] This domain is found abutting the CBM48 domain in the AtBE structure (**Figure 5**). Quixotically, this domain is located more than 40 Å from the active site in AtBE. It is, at this point, a mystery as to how removal of this domain alters the branch chain specificity of EcBE.

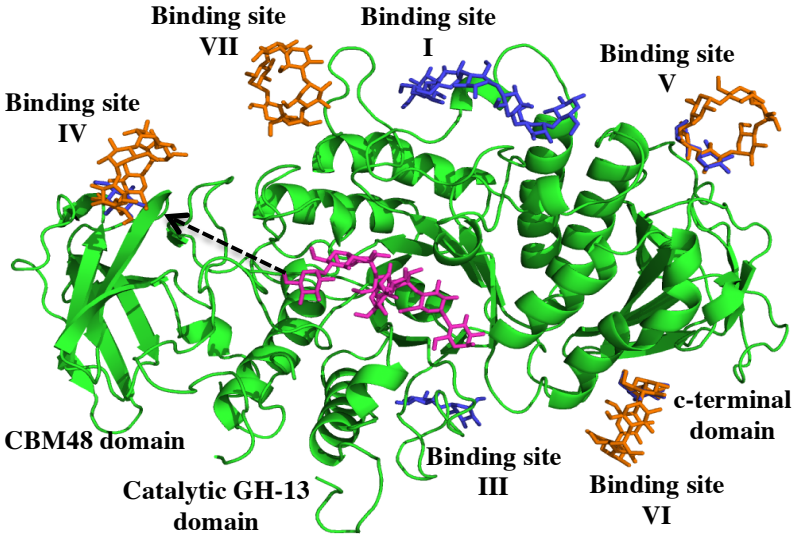

Figure 4. Composite structure of EcBE. All the bound-M7 molecules were overlayed to a single monomer since all binding sites were not occupied in all four molecules in the asymmetric unit due to crystal packing. α–CD's, orange, M7's, blue. A glucan was modeled in the active site based on the structure of a pullulanase (PDB 2FHF).

While BE exists as a single polypeptide in most glycogen-producing organisms (bacteria, fungi, higher mammals, etc.) two major classes of the enzyme are found in most plants, starch branching enzyme SBEI and SBEII. Two forms of SBEII are found in monocots, SBEIIa and SBEIIb. These enzymes differ in their substrate and branch chain length specificity.[23] Suppression of SBEIIb in maize and rice results in the "amylose-extender" phenotype, which allows the amylose content to grow to 50-90% (it is only 25% in the wild type plant).[2b, 24] On the other hand, suppression of SBEIIb had no notable effect on the morphology or structure of the starch granule in wheat, but simultaneous suppression of both SBEIIa and SBEIIb resulted in significant increases in amylose and profound effects on starch granule morphology. SBEII preferentially transfers shorter chains predominantly of lengths of 7 and 11, though there are significant populations of chains between these values.[25] SBEI, on the other hand, transfers much longer chains.[26] Studies of chimeric forms of SBEI and SBEII from maize have established the N-terminal domain to be important for both the substrate specificity and chain length specificity of these enzymes.[26b]

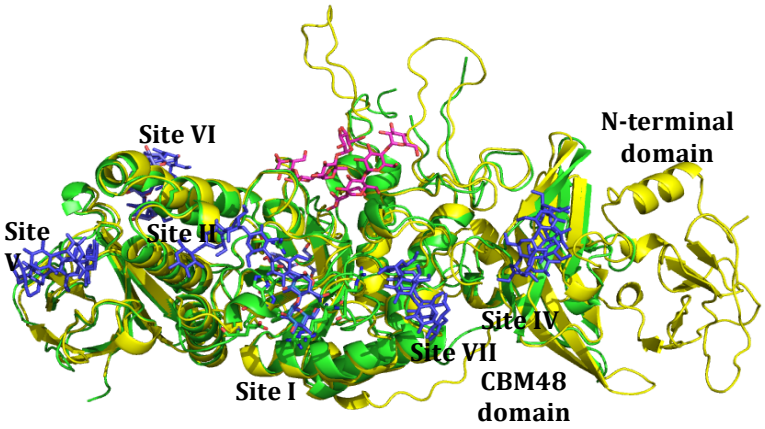

Figure 5. Overlay of the n113 EcBE (green ribbon) and MtBE (yellow ribbon) structures, showing the location of the n-terminal domain and binding sites (blue sticks).

A recent study has determined the kinetic parameters, substrate specificities and chain transfer preferences for all three of the rice SBE isoforms, SBEI, SBEIIa and SBEIIb. It was shown that none of the isozymes have significant activity toward amylose with short average glucan chain lengths (Degree of polymerization, DP) in the rage of 60. On the other hand, all isoforms were quite active against an amylopectin substrate, even though the average chain length was only 12-14 glucan units. More striking were the stark differences in chain length specificity of the enzymes. SBEIIb transfers almost exclusively chains of 6 or 7 glucan units, making it unusually specific for a BE. SBEI, transfers a much broader range of chains, ranging from shorter chains of 6-12 to much longer chains of 20-38. SBEIIa specificity was intermediate between the two, transfering a range of shorter chains from 6-15 and extending to much longer changes as long as 25-38. Very similar transfer chain preferences were seen in all isozymes whether the substrate was amylopectin or various sizes of amylose (DPn317 or DPn6510). The authors hypothesized that each SBE has a specific function in the formation of the lamellae of the starch granule, beginning with SSIIIa elongating a single branch from the preceding cluster. This is followed by the transfer of outer chains from the previous cluster to the elongating chain, requiring SBEI's ability to transfer much longer chains. This is then followed by formation of short, DP 6-7 chains by BEIIb which are then elongated by SSI and SSIIa to complete the formation of the new cluster.[23] This is consistent with the fact that an SBEIIb deficient mutant has no branches between the amorphous and crystalline lamellae and is significantly less branched than wild type starch. If true, this model predicts that SBEI would need traverse one cluster after the first step of the reaction, with a long glucan chain covalently attached to the enzyme, before it transferred this chain to the elongated chain on the opposite end. All of this leads us to the conclusion that the different reactivity and selectivity of the isoforms

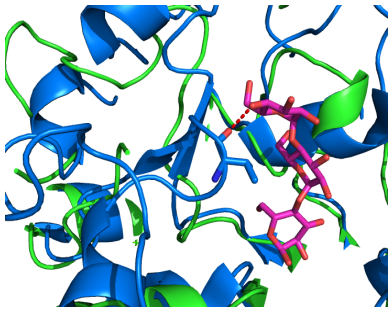

Figure 6. Differences in binding between EcBE and other GH13 enzymes. The structure of EcBE (molecule B, green) and α-amylase from pig pancreas (PDB 1UA3), yellow, maltotriose from this structure is shown, with carbons colored yellow and oxygens, red) are overlaid.

of plant SBEs represent physiologically relevant differences that are critical to the normal production of the starch granule in plants. Further, it is clear that the same isoform in different organisms may also have distinct reactivity profiles that are critical to the development of their particular starch.

Our structure of *E. coli* BE shed considerable light on the function of this enzyme.[7] We were able to see the similarities between the active site of our enzyme and the active sites of all of the other enzymes of this family whose structures are known. Furthermore, we were able to use this information to model a pentaglucan derivative of acarbose, which is a potent inhibitor of most of the other members of this family, in the active site. Acarbose, the most common inhibitor for GH13 hydrolases, does not inhibit BE from any organism. However, if the linked hydroxymethyl conduritol and 4-amino-4-deoxy-D-chinovose units are extended with sugars 7-20 glucose units in length on both sides, a potent inhibitor of BE, in fact the only known inhibitor of the enzyme (Bay e4609), is produced. Thus a relatively long glucan chain is required to tightly interact with the BE active site.[27] Our structure provides a possible explanation for the lack of binding of shorter glucans. The pentasaccharide modeled in the active site of BE shows that while the sugar moiety bound in the -1 subsite, which represents the site of the sugar that will be covalently linked to the enzyme and transfered to the 1,6 position of the substrate, is very similar to that seen for the other members of the amylase family whose structures are known, none of the residues that make up any of the other subsite binding sites are conserved. In fact even the loops that predominantly make up these subsites are not present in ECGE, indicating that BE does not bind glucans in these subsites. It thus appears that BE interacts with its maltodextrin substrate in a completely different way than that seen in any of the other family members whose structures are known. In fact there are now structures of several α–amylases, several exo-amylases, an isoamylase, several cyclodextrin glucanotransferase structures, and a few pullulanse structures. Though several of these structures show maltodextrins bound in the active sites, and virtually all show some resemblance to the sugar binding seen in the α-amylases, the loops about the active site that make up these binding sites are quite different from that seen in the structure of BE. As an example α-amylase from barley is shown overlayed on EcBE showing the loops that interact with the substrate (**Figure 6**). This may indicate that glucan binding may be more decentralized in BE, with the sugar binding distributed over a larger area than in the other enzymes in this family. Indeed only BE has the task of somehow measuring the length of the relatively long glucan chain to be transferred. Its tendency is to prefer lengths of at least 9-12 residues, but in some enzymes this preference can be much longer, as long as 20-30 residues. In addition BE from both maize and *E. coli* will not cleave chains that are less than 12 residues long.[26a]

We have taken the first steps in understanding this phenomenon in the previous grant period by determining structures of EcBE bound to a number of maltodextrins to get an idea of how EcBE interacts with its substrate. The hope was that these studies would not only shed light on how EcBE interacts with its glucan but would also give some idea of how BEs from plant sources might associate with their substrates. Though extensive co-crystallization trials were attempted with a wide assortment of maltodextrins, crystals of the complex could not be obtained. Finally, by systematically screening crystals of apo-EcBE in a wide range of soaking conditions, potential ligands and soaking times, we have been able to determine a variety of BE/maltodextrin complex structures. These structures have identified a variety of glucan binding sites on the surface of the enzyme. So far we have complexes with α-, β- and γ–cyclodextrins, maltose, M6 and maltoheptaose (M7). Together these structures identify and often serve to verify seven distinct binding sites on the surface of the enzyme. These sites are found over a wide region of the enzyme, but four of the seven sites are aligned on one face of the enzyme (**Figure 4**). Two sites (site V and site VI) make interactions with residues in both the catalytic and c-terminal Greek key domain. Several reports have shown this domain to be important for substrate recognition in branching enzymes.[22a, 26b, 28] Four of the sites (sites I, II and VII) are located on the catalytic domain. Two of these sites bind exclusively linear saccharides (sites I, II and III). The fourth catalytic domain binding site (site VII) exclusively binds cyclodextrins. All the rest of the sites bind both linear and cyclic maltodextrins.

Strangely, in spite of the fact that we have produced 4 linear oligosaccharide bound structures (maltotetraose, maltopentaose, M6 and M7 all show some oligosaccharide binding) and 3 cyclic oligosaccharide-bound structures, in none of these do we see any glucan binding in the active site. It is important to realize that the active site is completely unencumbered by crystal packing interactions *in all four molecules* of the asymmetric unit, making it unlikely that this is due to crystal packing interference. The fact that the enzyme is active may also play a role in this as active site binding may lead to reaction and dissociation. However, all of the glucans used in these studies display no activity for EcBE as they are far too short, and most of the other GH13 enzymes whose active-site-bound structures have been determined were also determined with active enzyme, so we do not believe that this explains the phenomenon, though we will test this in the upcoming grant period.[2, 21] We believe that the most likely explanation is that none of our glucans were long enough to properly mimic the bonafide substrate or product for branching enzyme. It is clear from a number of studies that EcBE only works on glucans at least 10 units long, and probably much longer. Furthermore, it transfers no chains shorter than 6 glucans, and most BEs transfer these very poorly if it transfers them at all. As will be described below, this hypothesis can also now be tested using homogeneous maltodextrins that are much longer than those previously available.

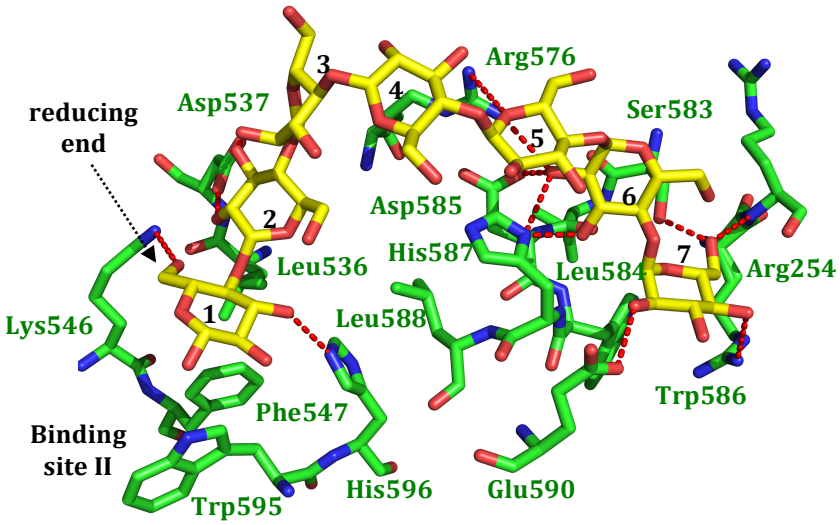

Figure 7. Binding sites I and II spanned by a single M7. M7, yellow, EcBE binding residues, colored by atom.

An intriguing binding site is binding site I, located in the center of the ridgeline of binding sites found on the top face of the enzyme (**Figure 4**). Both M6 and M7 bind to this binding site, though M6 binds worse and cannot reach all of the sugar binding sites. None of the cyclodextrins bind in this site as the geometry of these binding sites is inconsistent with a cyclic sugar (**Figure 7**). M7 can bridge this site and site II, with its non-reducing end occupying site I and its reducing end occupying site II. Several of the bridging sugars adopt relatively strained conformations, indicating that some strain is necessary to reach the two sites with a single 7 membered glucan chain. To get an idea of the length of the glucan that would be required to reach the active site, we have modeled polyglucan chains that reach from these binding sites to the -1 catalytic subsite conserved in our structure and found that it takes about 10 residues to reach either site, making these sites possibilities for binding at the end of the chain to be tranfered.[22, 28] The fact that site I specifically recognizes a non-reducing end of a glucan makes it the more likely candidate of the two sites. However, neither of these sites are conserved in SBEs or eukaryotic BEs, which would mean that donor strand binding is distinct in BEs.

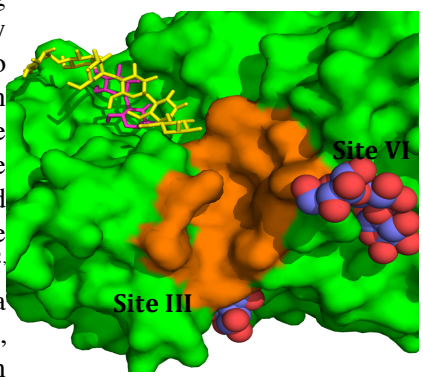

Figure 8. Surface depiction of the groove (orange) linking the active site with site III and IV. Site III and IV glucans, blue (C atoms and red(O atoms). Donor chain (magenta) and acceptor chain (yellow) glucans modeled in active site are shown.

Binding site III lies on the opposite face of EcBE. It is about 24 Å from the active site and would require 8 glucan residues to reach. A groove in the structure runs from this binding site to the active site (**Figure 8**). When a glucan is modeled in the active site, the reducing end of the pre-cleaved glucan would exit this groove.

Electron density can be found in this groove in molecule B of the M7-bound structure, though it is not of sufficient quality to identify. There is also electron density located above Tyr300 in the active site in this molecule, in the position expected for the -1 sugar, which is the sugar that is covalently attached to Asp405 in the first step of the reaction. This is the most highly conserved glucan binding site identified in our sites. Of the five residues that make direct contact with the glucan, three (P469, G476, and F477) are absolutely conserved in all species. N518 is only conserved in bacteria, and is replaced by a Lys in all higher organisms. Inspection of the structures of both EcBE and rice SBEI (rSBEI) shows that the Lys is fully capable of making equivalent interactions. The other residue, W478, provides a stacking interaction with the first glucose moiety in the binding site and is replaced by an Asp in bacteria and a Ser in higher organisms. However, the residue next to this residue (Gly447 in EcBE) is a His in virtually all plants and *Mycobacterium turberculosis*, and is positioned in both the MtBE and rSBEI structures to provide an equivalent stacking interaction. The glucan's non-reducing end is pointing towards the groove leading to the active site, consistent with the idea that the donor chain could run through this groove to this binding site prior to cleavage in the active site. Located very close to binding site III is binding site VI (**Figure 8**). It is located on the lip of the groove connecting the active site with Binding site III. Though well conserved in bacteria, this site is not conserved in plants or higher eukaryotes. It is bound by both linear and cyclic glucans, and in both cases the non-reducing end points toward the active site. Therefore both of these sites are not oriented correctly to be binding sites for the chain to be transferred. This is not surprising because in all GH13 enzymes the glucan chain to be cleaved runs in the same direction, with its non-reducing end exiting the opposite side of the active site relative to these binding sites (ref to PDB 1A47 for example).[29]

Binding site IV is located in the CBM48 domain. There is one other structure of a CBM48 domain, from the protein AMP-activated protein kinase (AMPk), a metabolic stress-sensing protein kinase.[30] The oligosaccharide binding in EcBE is very similar to that seen in AMPk. Two of the residues that interact with the glucans (Trp 159 and Lys 189) are conserved in these two proteins (Trp100 and Lys 126 in AMPk) and make similar interactions in each, with the Trp providing a hydrophobic platform for one glucan ring and the Lys making interactions with a 2-hydroxyl group of the γ-CD bound in the site (**Figure 9**). These two residues are absolutely conserved in all BEs, strongly suggesting that all of them bind glucans in this fashion. A comparison of the structure of MtBE with EcBE shows a substantial difference in the orientation of this domain relative to the catalytic domain and also shows an alteration in the structure of the Glucan binding site (**Figure 9**). We hypothesize that the loss of this domain in EcBE and the concomitant alteration of this binding site may play a role in the altered branch chain specificity of n113EcBE relative to the full length enzyme. This hypothesis will be tested in the next grant period.

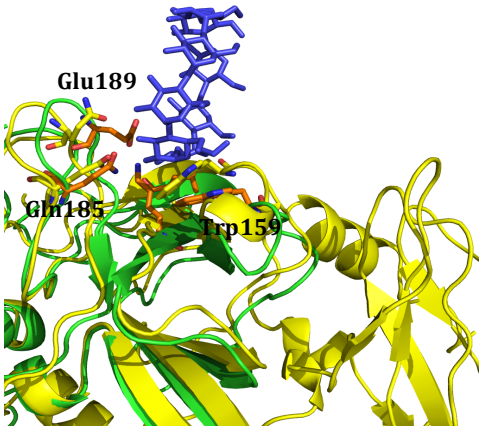

Figure 9. Overlay of MtBE and γ-CDEcBe at site IV. MtBE ribbon and residues (Gln224, Glu220, Trp168) are yellow. Note MtBE Trp168 collides with γ-CD. EcBe is green with orange highlighted residues.

When considered together, a working model for how BE might work can be considered. The two downstream binding sites (binding sites III and VI) are likely involved in binding the glucan chain on the reducing end of the glucan. Perhaps one of the sites binds the donor glucan, i.e. the glucan chain that is cleaved in the first step of the reaction, and the other binds the acceptor glucan, holding and directing it to the active site to accept the branch in the second step of the reaction (**Figure 8**). Since the acceptor glucan must approach from the top of the active site, binding site VI seems better positioned to fulfill this role while binding site III is better located to bind the reducing end of the donor glucan, directing it to the active site for cleavage. However, the large flexibility of the glucan chains make it possible that the roles could be reversed. Alternatively it is possible that both the donor and acceptor glucans bind the same site, as the residual chain must almost certainly exit the

active site prior to binding and reaction with the donor chain. The location, and especially the terminus of the chain to be transferred, must be located on the other side of the enzyme, where the non-reducing end of the chain exits the active site. There are several binding sites that could play this role, but binding sites I, II, IV and VII are the better positioned for this role than binding site V, which is much further away on the C-terminal domain and is not readily accessible to the active site (**Figure 4**). Binding site II has its reducing end pointing toward the active site, making it an unlikely candidate. Binding site VII is specific for cyclodextrins and depends on aromatic stacking interactions by two sequential aromatic residues (F261 and W262) on both sides of a glucose unit for its binding. These residues reside on a loop that is not well conserved, even in bacterial enzymes. In both the structures of MtBE and SBEI this loop is completely different than that seen in EcBE.[4, 19a, 19d] For these reasons binding site VII is unlikely to be responsible for the chain length specificity of EcBE. Though binding site I binds the non-reducing end of the sugar and directs the glucan toward the active site, it is also not strongly conserved, even in bacteria. This leaves only binding site IV, located on the CBM48 domain. This binding site shows very strong conservation, with three of the five residues conserved in bacteria and plants (W159, K189 and Q211) and a fourth, E215, identical it EcBE in all plants and a Gln in bacteria. A groove in the enzyme runs from the active site to this binding site (**Figure 4** shows a blue arrow in this groove). A maltodextrin stretched from the active site to this binding site requires 10-12 glucan units, consistent with the branch chain preference of EcBE. If this was the binding site for the transferred chain, the structural effects on this site caused by removal of the N-terminal domain could then explain the differences in branch chain specificity of the n112EcBE compared to the WT enzyme.

In the final grant period we have extended these studies to produce structures of EcBE bound to a maltooctaose and found the binding to be significantly different than what we had seen previously. In this structure, the glucan binds simultaneously to both binding site 1 and site VII. The longer oligosaccharide is sufficient to bridge these two sites. No binding is seen in site II, but binding is seen in sites III, IV, V and VII. We have also conducted a mutagenesis study of all VII binding sites and have found that site I and VI are both critical to the activity of the enzyme, while the other sites affect it's activity as well. This indicates that the sites identified are in fact functionally relevant. Preliminary transfer chain length assays are showing that some of these sites affect the length of chain transferred, however these studies are still ongoing.

In the final grant period we have also developed a novel crystallization of rice Starch Branching Enzyme I (sBE1). This has allowed us to produce a structure of sBE1 bound to maltododecaose. This structure clearly shows the malto-oligosaccharide binding at the edge of the catalytic domain and traversing the surface and almost binding within the active site of the enzyme. It's reducing end is in the direction of the binding site as expected for the transferred chain. We believe that this structure, shows, for the first time, how a donor oligosaccharide binds a branching enzyme and determines the branch chain specificity.

There is significant recent interest in genetic modification of crops to alter both the content and properties of the harvested starch. So far, however, most of these efforts have involved down regulation of the expression of individual biosynthetic enzymes. These have led to amylose-free (or waxy) starch obtained by antisense inhibition of GBSS and amylose-rich starch obtained by suppression of SBE isozymes.[3b, 31] However, it has not been possible to rationally alter the characteristics of these enzymes. The studies we propose will give us the critical, fundamental molecular-level understanding of these enzymes required to produce important useful variants. These redesigned enzymes will provide a powerful tool for better understanding the precise role each plays in the production of the starch granule. They will also enable the production of more diverse starches, leading eventually to more valuable starch-based products.

Publications from the previous grant period.

E. coli Glycogen synthase structures:

1. Sheng, F.; Jia, X. F.; Yep, A.; Preiss, J.; Geiger, J. H., The Crystal Structures of the Open and Catalytically Competent Closed Conformation of Escherichia coli Glycogen Synthase. *Journal of Biological Chemistry* **2009,** 284, (26), 17796-17807.
2. Sheng, F.; Yep, A.; Feng, L.; Preiss, J.; Geiger, J. H., Oligosaccharide Binding in Escherichia coli Glycogen Synthase. *Biochemistry* **2009,** 48, (42), 10089-10097.
E. coli branching enzyme
3 A manuscript describing the EcBE/M7 structure described above has bee submitted for publication to *Biochemistry*.
4 We will also submit a paper describing the structures of EcBE bound to α-, β– and γ- cyclodextrins in the near future as these structures are now complete.

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