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The structure-function relationships of maize starch synthase

by

Zhong Gao

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Physiology

Program of Study Committee:
Richard Shibles, Co-major Professor
Hanping Guan, Co-major Professor
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Iowa State University
Ames, Iowa
2001

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has met the dissertation requirements of Iowa State University

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For the Major Program

For the Major Department
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Chapter 1. General Introduction

Dissertation Organization

This dissertation reports on studies conducted on structure-function relationships of maize (Zea Mays L.) starch synthase IIa (SSIIa), and consists of three manuscripts that eventually will be submitted for publication in scientific research journals. The first manuscript (Chapter 2) is entitled “Lysine-193 of the conserved “K-X-G-G” motif is involved in enzyme catalysis rather than ADP-glucose binding of maize starch synthase IIa” by Zhong Gao, Peter Keeling, Richard Shibles, and Hanping Guan, and is to be submitted to Biochemistry. Chapter 3 is the second manuscript, entitled “Involvement of the conserved lysine-497 in substrate ADP-glucose binding of maize starch synthase IIa” by Zhong Gao, Peter Keeling, and Hanping Guan, and is to be submitted to Biochemistry. Chapter 4 is the third manuscript, entitled “Citrate influences the catalytic chain elongation specificity of maize starch synthase IIa” by Zhong Gao, Peter Keeling, and Hanping Guan, and is to be submitted to Archives of Biochemistry and Biophysics. Chapter 5 includes a general discussion and conclusions.

Literature Review

Starch is the most important form of carbon reserve in plants due to its significant contribution to human nutrition, and its commercial value. Starch is used for the production of processed foods, confectionaries, fruit products and beverages. The traditional non-food applications are in the paper industry, fermentation, chemical industry, and binders and adhesives industry. With industry demand for specialized starch increasing, significant efforts are being undertaken to improve starch quantity and quality.
This review mainly focuses on starch biosynthesis in storage organs, particularly in maize (Zea mays L.) endosperm. Starch is a widely distributed plant carbon reserve and an end product of photosynthesis. In photosynthetic cells, transit starch, functioning as temporary carbon storage, is accumulated in the process of active photosynthesis during the day, and degraded by respiration in darkness (Preiss and Sivak, 1996). Starch remobilization in photosynthetic cells can transport photosynthetic products to the whole plant. Starch is degraded to sucrose during darkness, and then sucrose is transported to the storage organs (such as maize endosperm) for resynthesis of starch. Hence, starch synthesis and degradation in the photosynthetic tissues are more dynamic than metabolism in the storage organs. The major difference in starch biosynthesis between photosynthetic organs and storage organs is that most chloroplasts in photosynthetic tissues are bioenergetically autonomous, whereas metabolism in storage organs largely depends on the supply of carbon precursors, reductant, and ATP. Consequently, this difference complicates the biosynthetic mechanism of starch in storage organs. For instance, it is clear that starch biosynthetic enzymes, such as ADP-glucose pyrophosphorylase, starch synthases, and branching enzymes are exclusively present in chloroplasts (Echeverria and Boyer, 1986). However, in storage organs, whether ADP-glucose pyrophosphorylase is located in plastid or cytosol is a subject of controversy. Moreover, it seems that metabolite transport between different compartments is essential for starch biosynthesis in storage organs. However, the mechanism is not fully understood yet (Emes and Neuhaus, 1997 and references therein).

**Starch biosynthesis pathway and its regulation**

In the past several decades, the efforts on characterizing biochemical lesions of plant starch mutants have greatly improved our understanding of the starch biosynthesis pathway.
Four major enzymes have been identified to be involved in starch biosynthesis, i.e., ADP-glucose pyrophosphorylase (AGPase, E.C.2.7.7.27), starch synthases (SS, E.C.2.4.1.21), branching enzymes (BE, E.C.2.4.1.18), and debranching enzyme (DBE) (Fig. 1, Smith et al., 1997).

The mutants related to starch biosynthesis are summarized in Table 1. Most mutants included in Table 1 are related to maize starch biosynthesis. Two mutants rug-5 and sta-3 from pea (*Pisum sativum* L.) and *Chlamydomonas reinhardtii*, respectively, are included to enrich information with respect to the soluble starch synthases. In terms of the process of starch biosynthesis, these mutants can be placed into four classes as follows:

1. Mutants affecting the supply of substrate, such as *mnl*, *btl*, *bt2*, *shl*, and *sh2*.
2. Mutants affecting starch synthases, such as *waxy*, *dull-1*, *rug-5*, *sugary-2*, and *sta-3*.
3. Mutants affecting starch branching enzymes, such as *ae*.
4. Mutants affecting starch debranching enzymes, such as *sugary-1*.

This section briefly discusses the above four processes and the related enzymes.

1. *Generation of ADP-glucose*

   It has been generally accepted that ADP-glucose is the direct substrate of starch biosynthesis, and the primary precursor of ADP-glucose is sucrose translocated to the maize endosperm. Characterization of the mutants *mnl*, *btl*, *bt2*, *shl*, and *sh2* may outline the framework of ADP-glucose synthesis. Maize mutant *mnl*, which lacks invertase activity (E.C.3.2.1.26) in the developing seed, results in about 60% reduction of soluble sugars in
Figure 1. starch biosynthesis pathway (Smith et al., 1997. Modified to show debranching enzymes in the conversion from ADP-glucose to amylopectin)
Table 1. Summary of plant mutants related to starch biosynthesis

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<th>Species</th>
<th>Biochemical lesion</th>
<th>Characterization</th>
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<tr>
<td><strong>Miniaturel (mnl)</strong></td>
<td>Maize</td>
<td>Invertase (Miller and Chourey, 1992)</td>
<td>The mutant lacks invertase activity in the developing seed. Soluble sugar decreases 60% in the mutant endosperm. (Lowe and Nelson, 1946; Miller and Chourey, 1992)</td>
</tr>
<tr>
<td><strong>Shrunken-1 (sh1)</strong></td>
<td>Maize</td>
<td>Sucrose synthase (Chourey and Nelson, 1976)</td>
<td>Starch content decreases 40–45% in the sh1 mutant seeds. Mutant seeds have a distinctive shrunken phenotype. (Chourey and Nelson, 1976, 1979; Chourey, 1981)</td>
</tr>
<tr>
<td><strong>Brittle-1 (bt1)</strong></td>
<td>Maize</td>
<td>Plastid membrane protein (putative adenylate translocator) (Heldt et al., 1991; Sullivan et al., 1991)</td>
<td>Starch content decreases 86% in the mutant seeds. The mutant endosperm development is significantly defective. (Nelson, 1988)</td>
</tr>
<tr>
<td><strong>Shrunken-2 (sh2)</strong></td>
<td>Maize</td>
<td>Large subunits of ADPGlc pyro-phosphorylase (Hannah and Nelson, 1975, 1976)</td>
<td>Mutant seeds produce only about 20% as much starch as do nonmutant seeds, and they are extremely defective. The developing endosperm of sh2 and bt2 has high concentration of sucrose and reducing sugars. The mutant lacks AGPase activity. (Tsai and Nelson, 1966; Nelson, 1988).</td>
</tr>
<tr>
<td><strong>Brittle-2 (bt2)</strong></td>
<td>Maize</td>
<td>Small subunits of ADPGlc pyro-phosphorylase (Hannah and Nelson, 1975, 1976; Okita et al., 1990; Smith-White and Preiss, 1992)</td>
<td>Mutant seeds contain very low percentage of amylose. Accordingly, the mutant lacks GBSS activity. (Sprague et al., 1943).</td>
</tr>
<tr>
<td><strong>waxy</strong></td>
<td>Maize</td>
<td>Granule-bound starch synthase (Nelson and Tsai, 1964)</td>
<td>The mutant seeds contain very low percentage of amylose. Accordingly, the mutant lacks GBSS activity. (Sprague et al., 1943).</td>
</tr>
</tbody>
</table>
Table 1. (continued)

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<th>Mutant name</th>
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<th>Characterization</th>
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<tr>
<td>dull-1</td>
<td>Maize</td>
<td>Soluble starch synthase II, branching enzyme (Gao et al., 1998)</td>
<td>Mutant starch has high degree of branching. There exist 15% intermediate materials. Mature kernel displays a tarnished, glassy, and dull phenotype. (Mangelsdorf, 1947; Wang et al 1993)</td>
</tr>
<tr>
<td>rug-5</td>
<td>Pea</td>
<td>Pea soluble starch synthase II (Craig et al., 1998)</td>
<td>The mutant has more very short chains (&lt;15 glucose units) and very long chains, and fewer intermediate chains (15-45 glucose), than wild type. Starch granule is grossly misshapen. (Craig et al., 1998)</td>
</tr>
<tr>
<td>sta-3</td>
<td>Chlamydomonas</td>
<td>Chlamydomonas soluble starch synthase (Fontaine et al., 1993)</td>
<td>The mutant amylopectin contains more very short chains and fewer longer chains. (Fontaine et al., 1993)</td>
</tr>
<tr>
<td>amylose extender (ae)</td>
<td>Maize</td>
<td>Maize branching enzyme Iib (Boyer and Preiss, 1978, 1981)</td>
<td>The mutant seeds contain a higher amylose content and anomalous amylopectin with fewer branches than nonmutant seeds. (Holder et al., 1974; Takeda et al., 1993).</td>
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endosperm and a grossly defective seed phenotype. It suggests that invertase is involved in sucrose transport into endosperm (Miller and Chourey, 1992). The nonmutant allele of shl encodes for sucrose synthase which converts sucrose to UDP-glucose and fructose (Chourey and Nelson, 1976). Characterization of the mutant bt1 identified a Pi translocator on the amyloplast envelope; this translocator may transport glucose-6-P, a precursor of ADP-glucose, into the amyloplast from the cytosol (Heldt et al., 1991; Sullivan et al., 1991). The mutants sh2 and bt2 cause the loss of ADP-glucose pyrophosphorylase activity in maize endosperm; the nonmutant alleles of sh2 and bt2 are the genes encoding for the larger subunit and the smaller subunit, respectively (Hannah and Nelson, 1975, 1976; Nelson, 1988; Okita et al., 1990). Based on these studies on maize starch mutants, a pathway of ADP-glucose synthesis is proposed (Smith et al., 1997. Fig.1).

ADP-glucose pyrophosphorylase is responsible for the synthesis of ADP-glucose, the glucose donor of starch biosynthesis. Elimination of activity of AGPase can severely reduce content of starch, suggesting that AGPase is the major route by which ADP-glucose is synthesized for starch biosynthesis (Tsai et al., 1966; Hannah et al., 1975; 1976). However, there is a controversy on where ADP-glucose is synthesized in cereal endosperms. Practically, this question is crucial for designing transgenic plants to improve the performance of AGPase. In photosynthetic cells, AGPase is located exclusively in plastids (Okita, 1992, and refs. therein). However, in maize endosperm location of AGPase and ADP-glucose synthesis is subject to controversy. Immunogold-labeling using antibodies raised to subunits of AGPase has shown that AGPase is plastidial in maize endosperm (Miller and Chourey, 1995). Also the plastids isolated from maize endosperm contain AGPase activity (Echeverria et al., 1988). On the other hand, there is evidence suggesting
that synthesis of ADP-glucose is in the cytosol. Absence of Brittle-1 protein, which is identified as a putative adenylate translocator located on the amyloplast envelope, results in a dramatic decrease in the rate of starch synthesis and an increase in the amount of ADPglucose in the endosperm (Cao et al., 1995; Shannon et al., 1996; 1998). It also has been shown that in vitro ADPglucose is transported into maize amyloplasts and incorporated into starch (ap Rees, 1995). Molecular characterization of AGPase has shown that the small subunit protein predicted from cDNA lacks a transit peptide (Giroux and Hannah, 1994). These results indicate that ADPglucose synthesis is cytosolic in storage organs. Denyer et al. (1996) investigated the location of AGPase in the developing maize endosperm using a plastid preparation, which led to the claim that there are distinct plastidial and cytosolic forms of AGPase and that the major form of AGPase in maize endosperm is extra-plastidial.

Plant AGPases are subject to allosteric regulation by an activator 3-phosphoglycerate (3-PGA) and an inhibitor inorganic phosphate (Pi) (Preiss, 1991). The physiological significance of the regulatory mechanism has been demonstrated by the observation that failure of AGPase to be activated by 3-PGA resulted in a starch deficient phenotype in a Chlamydomonas mutant (Ball et al., 1991). Moreover, based on a Kacser-Burns experiment, AGPase was regarded as a major site of regulation of starch synthesis (Neuhaus and Stitt, 1990), and activation of the enzyme by 3-PGA largely determined the rate of starch synthesis in vivo (Neuhaus et al., 1989).

The structure-function relationship of AGPase has been extensively studied from three aspects, i.e., subunit structure and function, substrate binding site, and activator binding site (Sivak and Preiss, 1998). It is well known that Shrunken-2 and Brittle-2 code for large subunits and small subunits, respectively. The plant native AGPases are tetrameric and
composed of two different subunits, a large subunit and a small subunit (Sivak and Preiss, 1998). The small subunit is primarily responsible for catalysis, whereas the large subunit mainly regulates the activity of small subunits by enhancing activator binding and decreasing inhibitor binding (Ballicora et al., 1996).

As for substrate binding of AGPase, lysine-195 has been identified as the binding site of \textit{E.coli} AGPase for the phosphate of glucose-1-P (Hill et al., 1991); and tyrosine-114 has been identified as involved in the binding of the adenosine portion of ATP in \textit{E.coli} AGPase (Lee and Preiss, 1986). Due to the high conservation of sequence between \textit{E. coli} and plant enzymes at the ATP and G-1-P binding sites, these sequences may also be important for the plant enzyme (Smith-White and Preiss, 1992).

Lysine-419 and lysine-382 have been identified as two binding sites for activator, 3-PGA, of AGPase in \textit{Anabaena}, using chemical modification and site-directed mutagenesis (Charg et al., 1994). Moreover, arginine-294 has been identified as the binding site for the inhibitor Pi of AGPase in \textit{Anabaena} (Sheng and Preiss, 1998). In potato, lysine-441 and lysine-417 have been identified as involved in binding of activator 3-PGA (Ballicora et al., 1996).

2. \textit{Starch synthase}

Starch synthases add glucosyl units at the non-reducing end of linear chains through new \( \alpha 1,4 \)-linkages, using ADP-glucose as the substrate. Starch synthases can be classified into two main classes, i.e. granule-bound starch synthase (GBSS) and soluble starch synthases, in terms of enzyme location and function. GBSS is mainly present in starch granules and responsible for catalyzing synthesis of amylose; soluble starch synthases are in the soluble phase of the stroma and are responsible for synthesis of amylopectin.
2.1 Granule-bound starch synthase (GBSS)

Granule bound starch synthase (GBSS) is associated with the granule. It is widely accepted that GBSS, also known as waxy protein, is responsible for synthesis of amylose (Tsai, 1974; Shure et al., 1983). The GBSS mutants, including rice (*Oryza sativa* L.) *waxy* mutant, maize *waxy* mutant, and potato (*Solanum tuberosum* L.) *amf* mutant, and antisense expression of GBSS in rice and potato, resulted in a dramatic reduction of amylose and corresponding loss of GBSS activity and absence of GBSS protein (MacDonald and Preiss, 1985; Sano, 1984; Hovenkamp-Hermelink et al., 1987; Kuipers et al., 1994). On the other hand, antisense GBSS expression in *Chlamydomonas reinhardtii* not only caused decreases in amylose content but also modified amylopectin structure, indicating that GBSS may also be involved in amylopectin synthesis (Delrue et al., 1992).

Smith et al. (1997) suggested that amylose biosynthesis be regulated through the availability of ADP-glucose to GBSS. The *Chlamydomonas* mutants *sta1* and *sta5* decrease starch content by 90% to 95%, and completely lack amylose; these mutants decrease the activities of AGPase and phosphoglucomutase (Van de Koornhuyes et al., 1996). Moreover, mutations at the *RB* locus of pea affect AGPase, and consequently reduce the percentage of amylose content in starch content (Bogracheva et al., 1995). These observations imply that the availability of the substrate ADP-glucose has more impact on GBSSI activity than other soluble isoforms, because GBSSI has a lower affinity for ADP-glucose, or because it is trapped inside the granule matrix (Smith et al., 1997).

2.2 Soluble starch synthases

Ozbun et al. (1971) found that there are two major forms of soluble starch synthase in maize endosperm, SSSI and SSSII, based on the elution profile from an anion
chromatography column. The biochemical properties were extensively characterized in Preiss’ laboratory (Preiss and Sivak, 1996). The major differences in biochemical properties between SSSI and SSSII are as follows.

(1) The maximal velocity of SSSI is greater with rabbit (*Oryctolagus cuniculus*) liver glycogen than with amylopectin; and the maximal velocity of SSSII is less with glycogen than with amylopectin (Preiss and Levi, 1980). This suggests that SSSI may have a higher preference for the short outer chains that are more prevalent in glycogen, whereas SSSII may have a higher preference for the longer outer chains more prevalent in amylopectin.

(2) SSSI is active without added primer in the presence of 0.5 M citrate, whereas SSSII is inactive (Preiss and Levi, 1980). However, a recent study showed that the unprimed activity of SSSI might be caused by contamination of primer in ADP-glucose (personal communication with Imparl-Radosevich, J. and Guan, H-P, 2000).

(3) SSSII is less sensitive to stimulation by citrate than SSSI (Preiss and Levi, 1980).

Hence, the first property may be the reliable parameter to distinguish SSSI and SSSII biochemically.

There are at least five genes coding for starch synthase in maize, including WX, DU1, zSSI, zSSIIa, and zSSIIb (Shure et al., 1983; Klosgen et al., 1986; Gao et al., 1998; Harn et al., 1998; Knight et al., 1998). WX encodes for GBSSI (Shure et al., 1983); DU1 may be responsible for the anion chromatography activity peak SSSII (but DU1 is designated as zSSIII because DU1 is the evolutionary counterpart of potato SSIH) (Gao et al., 1998; Cao et al., 1999); SSI is responsible for the activity peak SSSI (Knight et al., 1998; Imparl-Radosevich et al., 1998).
The genes coding for SSI, SSIIa, and SSIIb have been individually overexpressed in an *E. coli* strain lacking *glgA* and *glgB* in order to get rid of amylolytic enzymes and facilitate enzyme purification (Imparl-Radosevich et al., 1998; 1999). Characterization of recombinant SSI protein purified from *E. coli* has demonstrated that enzymatic properties of SSI are very similar to SSSI from developing maize endosperm (Imparl-Radosevich et al., 1998). The recombinant SSIIa differs from SSI and SSIIb in that SSIIa has a higher $V_{\text{max}}$ with amyllopectin than with glycogen in the presence of 0.5 M citrate, whereas SSI and SSIIb has a lower $V_{\text{max}}$ with amyllopectin than with glycogen in the presence of 0.5 M citrate (Imparl-Radosevich et al., 1998; 1999a). Hence, in the sense of primer preference, SSIIa is similar to SSSI activity peak. Cao et al. (1999) reported that DU1 (SSIII) and SSI most likely contribute to most of the soluble starch synthase activity in developing kernels, whereas SSIIa and SSIIb are expressed only at a low level. However, it should be pointed out that a low abundance enzyme may have a significant functional role in starch biosynthesis.

As compared with other starch biosynthetic enzymes, soluble starch synthase has fewer natural mutants. Therefore, it will be more difficult to understand the function of individual soluble starch synthase isoform in starch biosynthesis and granule biogenesis. So far in maize only the *dull1* mutant is related to soluble starch synthase (SSIII, responsible for SSSI activity peak). The most significant phenotype of this mutant is that *dull1* mutant has a higher percentage of amylose, contains 15% intermediate material, and has the highest degree of branching among normal or mutant kernels analyzed (Inouchi et al., 1987; Wang et al., 1993). Because of the pleiotropic effects of the *dull1* mutation, it is hard to differentiate the primary and secondary effects and determine the role of DU1 (SSIII). A possible
explanation is that SSIII (DUI) is responsible for the synthesis of intermediate chains in amylopectin; therefore absence of SSIII in the mutant leads to an increase of short chains and reduction of intermediate chains. There are several cases similar to dulll in other species. Fontaine et al. (1993) reported that the Chlamydomonas reinhardtii mutant sta-3, lacking SSSH activity peak, resulted in a decrease in number of intermediate size glucans and an increase in very short chains. Another mutant defective for SSSH activity, pea rug5, produced amylopectin containing fewer chains of intermediate length and more very short and very long chains (Craig et al., 1998). It is noteworthy that maize DUI (SSIII), Chlamydomonas reinhardtii SSII, and pea SSII are SSSH-activity-peak-like soluble starch synthases, and absence of SSSH-like starch synthases leads to a similar mutant phenotype.

Functionally, SSIIa seems an SSSH-like starch synthase because $V_{\text{max}}$ of SSIIa is less with glycogen than with amylopectin in the presence of 0.5 M citrate. But, more evidence is needed to extrapolate the function of DUI (or Chlamydomonas reinhardtii SSII, or pea SSII) to SSIIa (under certain conditions).

So far, not much is known about the structure-function relationship of starch synthases. Amino acid sequence alignments show that soluble starch synthase SSI, SSIIa, and SSIIb have an N-terminal extension (Dry et al., 1992; Edwards et al., 1995; Edwards et al., 1996; Harn et al., 1998). The “flexible” N-terminal extension has been shown to be unnecessary for catalysis involving soluble starch synthases (Edwards et al, 1996; Imparl-Radosevich et al., 1998, 1999b). The possible role of the N-terminal extension is proposed to be involved in determining starch synthase affinity for primers, or interacting with other enzymes, or chain elongation specificity, or binding of the enzyme to the granule (Dry et al., 1992; Imparl-Radosevich et al., 1998; 1999b).
Efforts have been devoted to identifying amino acids important for starch synthase catalysis and substrate binding. Chemical modification of SSIIa with phenylglyoxal (arginine-specific reagent) showed that arginine may be involved in the interaction of ADP-glucose with zSSIIa; however, no specific conserved arginine residue could be identified to be involved in ADP-glucose binding of SSIIa (Imparl-Radosevich et al., 1999a). Moreover, aspartate and glutamate residues have been shown to be involved in ADP-glucose binding (Nichols et al., 2000).

Furukawa et al. (1990; 1993) identified lysine-15 as the substrate ADP-glucose binding site of E. coli glycogen synthase using affinity labeling and site-directed mutagenesis. They proposed that the conserved K-X-G-G motif is involved in ADP-glucose binding of E. coli glycogen synthase. Because the K-X-G-G motif is highly conserved between E. coli glycogen synthase and starch synthases from diverse resources, the conserved lysine is widely accepted as the putative substrate ADP-glucose binding site of starch synthase in plants (Mason-Gamer et al., 1998). But, so far, the conserved K-X-G-G motif has not been directly characterized for its possible function.

3. Branching Enzymes

Branching enzymes catalyze the reaction cleaving the α-(1,4) linkage, simultaneously forming new α-(1,6) branch points. There are three isoforms of branching enzyme, i.e., BEI, BEIIa, and BEIIb in maize endosperm (Guan and Preiss, 1993). BEIIa and BEIIb are very similar and the product of the same gene (Singh and Preiss, 1985). BEI had the highest activity in branching amylose; in contrast, BEIIa and BEIIb preferred amylopectin (Guan and Preiss, 1993). This suggests that BEI catalyzes the transfer of longer branched chains, whereas BEIIa and BEIIb catalyze the transfer of shorter chains. Therefore, individual BE
may have a distinct function in starch biosynthesis. BEI may be involved in the synthesis of lightly branched polysaccharides, and then BEII isoforms and starch synthase act on the lightly branched polysaccharides to synthesize amylopectin. Hence, BEI may be involved in synthesis of the interior (B) chains of amylopectin, whereas BEIIa and BEIIb may focus on synthesis of the exterior (A) chains (Sivak and Preiss, 1998).

Maize endosperm \textit{ae} mutation, rice \textit{ae} mutation, and pea embryo \textit{r} mutation result in a phenotype of higher amounts of amylose and a longer chain amylopectin (Boyer and Preiss, 1981; Bhattacharyya et al., 1990; Mizuno et al., 1993). The nonmutant allele \textit{ae} codes for BEIIb in maize endosperm (Fisher et al., 1996). Obviously, absence of BEIIa resulted in the reduction of the shorter exterior (A) chains. So far, no mutation affecting maize BEI has been found.

To understand the structure-function relationship of maize branching enzymes, Kuriki et al. (1997) constructed chimeric enzymes from the maize BEI and BEII. The chimeric enzyme \textit{mBEII-I BspH1}, consisting of the carboxyl-terminal part of \textit{mBEI} and amino-terminal part and central part of \textit{mBEII}, had properties different from both \textit{mBEI} and \textit{mBEII}. The chimeric enzyme was similar to \textit{mBEI} in preference for amylose, suggesting that the carboxyl-terminal part is involved in substrate specificity and catalytic capacity. On the other hand, the chimeric enzyme transferred shorter chains than \textit{mBEI}, similar to \textit{mBEII}, suggesting that the amino terminus of the maize BE is important for the polysaccharide chain length specificity.

Chemical modification by EDAC suggested that an acidic amino acid might be involved in BE catalysis (Kuriki et al., 1996). Based on sequence alignments of starch- and glycogen-branching enzymes, four conserved regions have been identified to be similar to
the putative catalytic domains of the α-amylase family of enzymes (Svensson, 1994). These four conserved regions are in the central portion of BE. Site-directed mutagenesis has shown that the conserved Asp of regions II and IV and the Glu residue of region III are important for BE catalysis (Kuriki et al., 1996).

4. Debranching enzymes

Debranching enzymes (DBE) catalyze the hydrolysis of α-(1,6) linkages. There are two isoforms, i.e., isoamylase-type DBEs and pullulanase-type DBEs. Isoamylase-type DBEs mainly hydrolyze α-(1,6) linkages in denatured amylopectin and glycogen, but do not cleave branches in pullulan. On the other hand, pullulanase-type DBEs hydrolyze pullulan or amylopectin, but are not active in cleaving glycogen (Lee and Whelan, 1971; Doehlert and Knutson, 1999; Manners, 1997). Both isoamylase-type DBE and pullulanase-type DBE have been biochemically identified in developing maize kernels (Pan and Nelson, 1984; Doehlert and Knutson 1991), and the genes coding for the two types of DBE in maize have been cloned (James et al., 1995; Beatty et al., 1999).

Characterization of maize mutant *sul*, Arabidopsis mutant *dbel*, Chlamydomonas mutant *sta7*, and rice mutant *sul* has demonstrated that alteration of a isoamylase-type DBE resulted in amylopectin deficiency and phytoglycogen accumulation, suggesting that isoamylase-type DBE is involved in amylopectin biosynthesis (Shannon and Garwood, 1984; James et al., 1995; Rahman et al., 1998; Zeeman et al., 1998; Mouille et al., 1996; Nakamura et al., 1996; Kubo et al., 1999). It appears likely that pullulanase-type participates in a degradative process. Little is known about the structure-function relationships of DBEs. The
role of DBEs in starch biosynthesis will be discussed in details in the section “What controls starch structure”.

Understanding of mechanism of starch biosynthesis has greatly facilitated efforts to improve starch production and quality using biotechnology. Genetic engineering has been applied for the enhancement of total starch production in transgenic potato plants (Stark et al., 1992). The improvement of starch quantity was achieved by transferring a mutant allele of *Escherichia coli* glgc, which is insensitive to allosteric regulation, into potato (Stark et al., 1992). As Hannah et al. (1993) emphasized, a deeper understanding of the starch biosynthesis pathway is essential to any genetic manipulation aimed at improvement of starch quantity and quality. Next, I will discuss two fundamental questions with respect to starch biosynthesis, i.e., (1) What controls the rate of starch biosynthesis?; (2) What controls starch structure?

**What controls the rate of starch biosynthesis in storage organs?**

Starch in the starch-storage organs, such as in potato tubers, legume embryos, and cereal endosperms, constitutes about 50 to 80% of the dry weight at maturity. Thus, understanding of starch biosynthesis and its regulation is very important for genetic manipulation of starch production. When biotechnologists are designing a certain scheme for genetic modification of the starch biosynthesis pathway in order to achieve more starch and novel starch structure, a basic question will certainly come out, i.e., what controls metabolic flux of starch biosynthesis and structure of starch? The regulation of starch biosynthesis in the starch storage organs of higher plants is very complex and poorly understood. To characterize flux control of a metabolic pathway, the basic task is to identify the controlling
site(s) (key enzyme or/and process) in the pathway. The flux-controlling site(s) and mechanism of starch biosynthesis in higher plants are still subject of controversy.

A widely accepted view of regulation of starch biosynthesis is that ADP-glucose pyrophosphorylase (AGPase) plays a major role in controlling metabolic flux of starch biosynthesis in storage organs (Stark et al., 1992). The supporting evidence includes that

1. AGPases are subject to allosteric regulation by 3-phosphoglycerate (3-PGA, an activator) and inorganic phosphate (an inhibitor) (Preiss, 1991). Also, it is believed that AGPases are effectively irreversible in vivo (Preiss, 1991). Hence, AGPase is regarded as a rate-limiting factor in starch biosynthesis pathway.

2. AGPase is much more important in controlling rate of starch biosynthesis than other enzymes in Arabidopsis chloroplasts (Neuhaus and Stitt, 1990).

3. A large reduction in AGPase decreased starch content in sh-2 and bt-2 mutants (Hannah and Nelson, 1975; 1976).

4. The sta-1 (sta-1 encodes one subunit of AGPase) Chlamydomonas mutant shows reduced sensitivity of AGPase to activation of 3-PGA. This mutation caused a 95% reduction of starch content, suggesting that the allosteric regulation property of AGPase is essential for starch biosynthesis (Ball et al., 1991; Van den Koornhuyse et al., 1996).

5. Transfer of mutant E.coli AGPase into potatoes can enhance starch biosynthesis in potato tubers (Stark et al., 1992). This mutant E. coli AGPase is less sensitive to allosteric regulators (3-PGA and Pi) than is wild type. This experiment is widely cited as a strong evidence for the claim that AGPase is a controlling factor in starch biosynthesis.
Smith et al. (1995) questioned and challenged the first three supporting evidences in their review. Smith pointed out, in another review published two years later, that flux through starch biosynthesis pathway may be regulated in different ways due to variation in role of AGPase between different plant organs (Smith et al., 1997).

Smith et al. (1995) suggested that metabolic control analysis be applied to analyze the relationship between the activity of an enzyme and metabolic flux in order to determine the importance of the enzyme in flux control. Denyer et al. (1995) estimated the contribution of AGPase and branching enzyme to control of starch synthesis in developing pea embryos. They reported that AGPase and branching enzyme have low flux control strength. Singletary et al. (1997) also applied the flux control method (Kacser and Burns, 1973) to estimate flux control strengths of specific enzymes using maize starch deficient mutants. Their results indicated that branching enzyme, ADPglucose pyrophosphorylase, and sucrose synthase had only minimal control over starch biosynthesis, implying that AGPase does not control flux in starch biosynthesis in maize endosperms. These researches imply that other starch biosynthetic enzyme(s) instead of AGPase may be the controlling factor in maize starch biosynthesis. Smith et al. (1995) proposed that the soluble starch synthase might be more important than AGPase in controlling rate of starch biosynthesis in storage organs. This proposal was based on the observation that treatment of wheat (Triticum aestivum L.) ears and grains at high temperature decreased soluble activity of starch synthase and rate of starch synthesis simultaneously and to a similar degree (Jenner et al., 1993; Keeling et al., 1993). They reported that the dramatic reduction in starch synthesis might be primarily due to reduced activity of soluble starch synthase.
Interestingly, Sweetlove et al. (1999) estimated the control coefficient of AGPase on starch accumulation in potato tubers, and claimed that AGPase exerted considerable control over the flux to starch. In their experiment, the control coefficients of AGPase on starch accumulation in intact plants and tuber discs were estimated to be about 0.3 and 0.55, respectively. Also, it was shown in their research that reduction in the activity of AGPase in the potato tuber leads to a major redirection of carbon fluxes with increased flux to sucrose and decreased flux to starch.

Geigenberger et al. (1999) provided a very helpful example for further understanding of the matter. They investigated the contribution of AGPase to control of starch biosynthesis under water stress. The control coefficients of AGPase over starch synthesis were estimated to be 0.3 to 0.5 for non-stressed or moderately water-stressed potato tubers, compared with a control coefficient of 0.16 for severely water-stressed tubers. The significance of this research is that control by AGPase is subject to change in response to environmental factors.

In summary, there are two major views on what is the controlling step in starch biosynthesis, i.e., AGPase and soluble starch synthase. Significant variation in estimated control coefficients of AGPase among different species, as well as different organs, suggests that the role of AGPase in flux control in starch synthesis may be different between species and between organs (Smith et al., 1995). Furthermore, there is evidence that the contribution of certain flux controls may be subject to change with different environmental factors, and with different developmental stages (Geigenberger et al., 1999).

The weakness of supporting evidence for soluble starch synthase is that the contribution of soluble starch synthases to the control of starch biosynthesis cannot be estimated due to lack of effective mutants for control coefficient estimation. Though dulll
has been identified as a gene coding for soluble starch synthase (Gao et al., 1998), the control coefficient cannot be calculated because either the changes in enzyme or changes in flux were too small to reliably calculate a ratio for *dull-1*.

**What determines starch structure?**

Starch exists as granules composed of two types of polysaccharide: amylopectin and amylose. Amylopectin is a highly branched structure of relatively short chains, consisting of α-(1,4)-linked glucose residues with α-(1,6)-linkages branches. Amylose is a smaller and relatively unbranched polymer, consisting of long chains of α-(1,4)-linked glucose residues. Amylopectin accounts for 70 to 80% of most starches, and it is very important for granule structure and formation; amylose comprises about 20 to 30% of most storage starch. The starch granule has a very complex structure. The most accepted structure model is the cluster model (French, 1984). A unit amylopectin cluster is composed of the amorphous lamella and the crystalline. The conservation of the amylopectin cluster size (9nm) among different plant species suggests that there exists a well-conserved mechanism for determining this cluster size (Jenkins et al., 1993).

Martin and Smith (1995) proposed a model for the interaction of the starch biosynthetic enzymes. AGPase in the soluble phase of the amyloplasts supplies ADP-glucose to starch synthases. Soluble starch synthases are in the soluble phase and on the granule surface, elongating α-(1,4)-linked glucans. Branching enzymes catalyze the addition of α-(1,6)-branches to α-(1,4)-linked glucans. These α-(1,6)-branched polymers can then be elongated further by starch synthases. GBSS is active within the granule. Because the granule separates α-(1,4)-linked glucans synthesized by GBSS from branching enzymes in soluble phase, GBSS can synthesize amylose within the granule.
Smith et al. (1995) revised the above model based on several new observations. First, the endosperms of maize and wheat, and the embryos of pea, all have granule-bound isoforms of starch synthases in addition to waxy protein (MacDonald and Preiss, 1985; Denyer et al., 1993), and the starch of the waxy mutant of maize is amylose-free even though it retains non-Waxy starch synthase activity (Macdonald and Preiss, 1985). So, they speculated that these isoforms might interact with granule bound branching enzymes to contribute to amylopectin synthesis.

The above model represents the conventional view of starch synthesis: Starch synthases and starch branching enzymes determine the structure of amylopectin, and the branching pattern of amylopectin is determined by specificity of branching enzymes. In this conventional view, debranching enzyme is not thought to be directly involved in starch biosynthesis. However, comparison of natural structures between animal glycogen and plant starch raises a question: Why can plants achieve the crystalline structure but animals cannot. Although SS and BE in higher plants are functionally similar to those involved in bacterial glycogen synthesis, it has not been successful to synthesize starch with plant SS and BE in vitro or in E. coli. Guan et al. (1995) observed that both maize starch branching enzymes expressed, alone or in combination, in an E. coli strain lacking its own glycogen branching enzymes have generated glycogen-like products rather than granular starch. Phytoglycogen, a water-soluble polysaccharide with similar structure to glycogen, has been found in maize mutants sul, Chlamydomonas reinhardtii mutant sta7, Arabidopsis mutant DbeI, and rice sul mutant (Shannon et al., 1984; James et al., 1995; Rahman et al., 1998; Zeeman et al., 1998; Mouille et al., 1996; Nakamura et al., 1996; Kubo et al., 1999). Molecular characterization has found that these mutations affect the isoamylase-type debranching
enzyme (James et al., 1995). Therefore, debranching enzyme may play a direct role in starch biosynthesis.

Ball et al. (1996) proposed the glucan-trimming model for starch granule biogenesis. According to this model, both branching enzymes and debranching enzymes are essential for amylopectin synthesis. When the crystalline lamella has reached the critical size, the branching enzymes synthesize preamylopectin, a highly branched intermediate, using the constitutive glucans as substrate. Simultaneously, debranching enzymes will trim the loosely branched glucans of preamylopectin in order to generate the next planar crystalline lamella. In this model, a highly branched polysaccharide is regarded as the intermediate of amylopectin synthesis and granule biogenesis. As compared with the conventional model, the core of this model is the significant involvement of debranching enzyme in amylopectin granule biogenesis, i.e., debranching enzymes are essential for forming semi-crystalline structure. This view is in contrast to the conventional view that debranching enzymes are only involved in starch degradation.

The “Ball model” was challenged by the observation that Arabidopsis dbel mutants simultaneously accumulate phytoglycogen and starch granules in the same cells (Zeeman et al., 1998). Zeeman et al. (1998) proposed another model, “water-soluble polysaccharide (WSP)-clearing model”, in which debranching enzymes eliminate the products of non-productive pathways in the soluble phase. Starch synthases and branching enzyme are proposed to work both at the granule surface and in the stroma. In the stroma, SS and BE act on maltooligosaccharide (MOS) to produce WSPs, small branched glucans, which may serve as alternative substrates for SS and BE. On the other hand, at the granule surface, SS and BE synthesize amylopectin. In this model, the role of debranching enzyme is to prevent the non-
productive WSP synthesis in order to insure the amylopectin synthesis pathway at the granule surface. The “WSP clearing” model somehow is a new version of the conventional view of amylopectin synthesis in which the role of debranching enzymes focuses on degradation of products of nonproductive pathways instead of direct involvement in amylopectin biosynthesis and granule biogenesis.

Recently, Myers et al. (2000) revised the “glucan trimming model” in order to solve the contradictions with the observed phenomenon. The main modifications and expansions of the revised model are follows:

(1) Crystallization, functioning as a carbon sink, promotes amylopectin biosynthesis.

(2) It is still proposed that debranching enzyme is required for amylopectin biosynthesis and crystallization. However, the revised model specifies that a certain threshold level of DBE activity is required for initiation of glucans trimming. This expansion can explain the simultaneous occurrence of phytoglycogen and starch granules.

(3) Phytoglycogen and amylopectin are proposed as two possible end products of the diverging pathway. A DBE deficiency results in loss of the capacity to prevent continued elongation and branching of WSP, and finally causes accumulation of phytoglycogen. The revised model carefully uses “preamylopectin” rather than phytoglycogen to describe the intermediate in amylopectin synthesis and granule biogenesis. Preamylopectin is defined as any branched glucan still accessible to the solute in this revised model. Hence, in the revised model, phytoglycogen is regarded as an end product rather than an intermediate.

(4) The revised model speculates the possible involvement of disproportion enzyme (D-enzyme) in amylopectin synthesis.
Generally, the revised “glucan-trimming model” still emphasizes that debranching enzymes directly modify a precursor of amylopectin, which is the main difference between the revised model and the “WSP clearing model”.

Amylopectin biosynthesis and granule biogenesis are very sophisticated. It is most interesting to understand the biochemical mechanism of crystallization, i.e., transition from lower order structure to higher order structure. Now, the role of debranching enzymes in the crystallization process is debated. Further understanding depends on a greater understanding of the specific properties of starch synthesis enzymes, creation and characterization of new mutants as well as transgenic studies.

Rational and Objective for My Research

The work presented in this dissertation is aimed at investigating the structure-function relationships of soluble starch synthase and exploring the mechanisms of starch biosynthesis. Chapter 2 characterizes the function of the conserved lysine in the K-T-G-G motif, the widely accepted ADP-glucose binding site of starch synthases in plants. Chapter 3 identifies a possible ADP-glucose binding site of maize SSIIa, and proposes a possible catalytic mechanism of SSIIa. Chapter 4 investigates the citrate-induced switch of outer chain length preference of SSIIa.

References


Imparl-Radosevich J.M., Keeling P., and Guan H.P. (1999a) Essential arginine residues in maize starch synthase IIa are involved in both ADP-glucose and primer binding. FEBS letters 457: 357-362


Chapter 2.

Lysine 193 of the Conserved “K-X-G-G” Motif Is Involved In Enzyme Catalysis Rather Than ADP-glucose Binding of Maize Starch Synthase IIa

A paper to be submitted to Biochemistry
Zhong Gao, Peter Keeling, Richard Shibles, and Hanping Guan

ABSTRACT

Based on affinity labeling and site-directed mutagenesis, it has been suggested that the lysine residue in the conserved K-T-G-G motif could be the substrate ADP-glucose binding site of \textit{E.coli} glycogen synthase (GS) (Furukawa et al., 1990; 1993). Since the K-T/S-G-G motif is highly conserved between \textit{E.coli} GS and all the maize (\textit{Zea mays L.}) starch synthase (SS) isozymes, it has become widely accepted that the lysine residue in the conserved K-T-G-G motif of SS may also function as the substrate ADP-glucose binding site for maize SS as well. We have used chemical modification and site-directed mutagenesis to examine the proposed function of this lysine residue in SS. Chemical modification of maize SSIIa showed that pyridoxal-5’-phosphate inactivated maize SSIIa activity in a time and concentration dependent manner. Furthermore, ADP-glucose completely protected SSIIa from inactivation by pyridoxal-5’-phosphate, indicating that lysine residue(s) could be important for ADP-glucose binding and enzyme catalysis. To identify the specific lysine residue(s) important for ADP-glucose binding and catalysis, the lysine residue 193 in the conserved K-T-G-G motif was mutated, respectively to arginine (R), glutamine (Q), and glutamic acid (E). In contrast to \textit{E.coli} GS, mutation of conserved lysine193 (K-T-G-G) in maize did not alter the Km for ADP-glucose while significantly changing the enzyme
activity. Our results indicate that lysine-193 (K-T-G-G) is involved in enzyme catalysis rather than substrate ADP-glucose binding.

**INTRODUCTION**

Mechanisms of starch biosynthesis have been of recent interest due to the possibility of improving starch quantity and structure through biotechnology. Starch synthase, a major starch biosynthetic enzyme, catalyzes glucosyl transfer from ADP-glucose to the non-reducing end of α-glucan, determining starch structure in a concerted action with branching enzymes (BE) and debranching enzyme (DBE). Intensive efforts have been devoted to biochemical analysis of maize starch mutants and molecular cloning of the genes encoding for SS in maize endosperm. So far five SS genes have been identified and characterized in maize, i.e., granule bound starch synthase (GBSS) (Shure et al., 1983; Klosgen et al., 1986), DU1 (Gao et al., 1998), zSSI (Knight et al., 1998), zSSIIa, and zSSIIb (Harn et al., 1998). Although the precise pathway of starch biosynthesis and its regulation still remain to be elucidated, it has been proposed that soluble SS plays an important role in controlling the flux rate of starch biosynthesis in wheat (*Triticum aestivum* L.) and maize storage organs (Jenner et al., 1993; Keeling et al., 1993; Keeling et al., 1994). Therefore, starch synthase enzymes play an important role in starch biosynthesis.

Characterization of maize SS isozymes has demonstrated that each isoform has distinct catalytic properties and specificities in chain elongation. For instance, SSIIa has a higher activity with amylopectin as primer while SSI and SSIIb have a higher activity with glycogen in the presence of 0.5 M citrate (Imparl-Radosevich et al., 1998; 1999). Structurally, a significant characteristic of soluble starch synthases is that all maize soluble
starch synthases possess a divergent N-terminal extension which is absent in GBSS and *E.coli* GS (Knight et al., 1998; Harn et al., 1998). Arginine has been found to be essential to substrate ADP-glucose binding using chemical modification (Imparl-Radosevich et al., 1999). In addition, it has been shown that the conserved aspartate and glutamate residues could be involved in stabilizing ADP-glucose binding to SS (Nichols et al., 2000). However, in comparison with other starch biosynthetic enzymes, such as ADP-glucose pyrophosphorylase, the structure-function relationship of SS in substrate binding, catalysis and regulation is still poorly understood. Hence, a further characterization of the structure-function relationship of SS will certainly promote understanding of mechanisms of starch biosynthesis.

*Escherichia coli* GS and maize SS are functionally similar, catalyzing the formation of α-1,4-glucosidic linkage with ADPG as a substrate. Using affinity labeling and site-directed mutagenesis, Furukawa et al. (1990, 1993) have shown that lysine-15 in the conserved “KTGG” motif is directly involved in substrate ADPG binding of *E.coli* GS. Because the K-T-G-G motif is highly conserved between *E. coli* GS and SS from diverse resources (van de Leij et al., 1991; Preiss and Sivak, 1996; Cao et al., 1999), the conserved lysine residue of the “KTGG” motif has been widely regarded as the putative substrate ADP-glucose binding site of SS in higher plants (Baba et al., 1993; Marshall et al., 1996; Takaoka et al., 1997; Mason-Gamer et al., 1998). However, so far there is no direct experimental evidence to support or deny such a hypothesis.

We studied the function of the lysine residues in maize SSIIa, using chemical modification and site-directed mutagenesis. We demonstrate that lysine is functionally essential for ADP-glucose binding of starch synthase IIa; however, lysine-193 in the
conserved K-T-G-G motif, the widely accepted putative ADP-glucose binding site in higher plants, is actually involved in catalysis rather than substrate ADP-glucose binding in maize SS. This suggests that the lysine residue in the conserved K-T-G-G motif may function differently between bacteria and plants.

MATERIALS AND METHODS

Materials

Enzymes for DNA manipulation were obtained from New England Biolabs. Oligonucleotide primers for site-directed mutagenesis and DNA sequencing were synthesized and purified by the DNA sequencing facility at Iowa State University. ADP-[U-\textsuperscript{14}C] Glc was synthesized using [U-\textsuperscript{14}C]-glucose-1-phosphate (Amersham) and \textit{E.coli} ADP-Glc pyrophosphorylase as described (Espada, 1962). All other chemicals and supplies were purchased as the highest quality available from Sigma, or as otherwise indicated.

Chemical modification of maize SSII\textsubscript{a} with pyridoxal 5'-phosphate

A stock solution of pyridoxal 5'-phosphate (PLP, 20mM) was freshly prepared by dissolving PLP powder in 30 mM NaOH, and kept on ice. The purified SSII\textsubscript{a} enzyme (3 μM) was incubated with PLP in the dark at 37°C, in a solution containing 50mM Hepes (pH 7.8), 1mM EDTA, 1 mM DTT, and PLP (0 to 15mM) in a final volume of 200 μL. At defined time intervals, the incubation mixture was immediately chilled to 0°C, and sodium borohydride was added to a final concentration of 33mM to stop the reaction. After standing on ice for at least 15 minutes, aliquots of the sample were withdrawn, diluted (1:4) and assayed (5 μL) for enzyme activity. In the case of substrate protection experiments, the
purified SSIIa was incubated with 10mM PLP, in the absence or presence of 2mM ADP-glucose.

**Site-directed mutagenesis**

Site-directed mutagenesis was carried out on full-length maize SSIIa, N-terminally truncated maize SSIIa (SSIIa-2), and *E.coli* glycogen synthase (*glgA*) subcloned into the pET-21 expression vector (Novagen) (Fig. 1). Site-directed mutagenesis was performed using a PCR in vitro site-directed mutagenesis kit (Stratagene), following the manufacture’s manual. The primers used in site-directed mutagenesis are listed below. The products of the mutagenesis reactions were transformed into DH5α competent cells, individually, followed by DNA amplification and sequencing to confirm the presence of the desired mutations. To ensure that there was no undesired mutation introduced by PCR, the DNA fragment containing the desired mutation was re-subcloned into the wild type plasmids. The entire re-subcloned DNA fragments were then sequenced again.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Full-length SSIIa K193R</td>
<td>ExS 78</td>
<td>5'-GT TCT CCA TGG TGC AGA ACA GGT GGT C-3'</td>
</tr>
<tr>
<td>Full-length SSIIa K193Q</td>
<td>ExS 80</td>
<td>5'-GT TCT CCA TGG TGC CAA ACA GGT GGT C-3'</td>
</tr>
<tr>
<td>Full-length SSIIa K193E</td>
<td>ExS 165</td>
<td>5'-GT TCT CCA TGG TGC GAA ACA GGT GGT C-3'</td>
</tr>
<tr>
<td>Truncated SSIIa K19E</td>
<td>ExS 165</td>
<td>5'-GT TCT CCA TGG TGC GAA ACA GGT GGT C-3'</td>
</tr>
<tr>
<td><em>glgA</em> K15Q</td>
<td>ExS 220</td>
<td>5'-G TTC CCG CTG CTT CAA ACC GGC GGT CTG-3'</td>
</tr>
<tr>
<td><em>glgA</em> K15E</td>
<td>ExS 222</td>
<td>5'-G TTC CCG CTG CTT CAA ACC GGC GGT CTG-3'</td>
</tr>
</tbody>
</table>

**Expression and purification of the mutant enzymes**

Full-length SSIIa, N-terminally truncated SSIIa, glycogen synthase, and their mutant plasmids were individually transformed into *E.coli* mutant strain EX2 [*glgA*- and *glgB*-] that
allows the measurement of activities of mutant enzymes in the absence of a bacterial glycogen synthase and branching enzyme. Overnight cultures were diluted 1:20 into 10 L of LB broth and grown at 37 °C until OD<sub>600</sub> reached 0.6. Expression of SS enzymes was induced by the addition of 1 mM IPTG. The cultures were then grown at room temperature for 4 hours. The cell pellet was frozen in liquid nitrogen and stored at -70°C after harvesting by centrifugation at 9600g for 10 minutes.

Full-length maize SSIIa, N-terminally truncated maize SSIIa, <i>E.coli</i> glycogen synthase and their mutants were purified to apparent homogeneity with a procedure similar to that described previously (Imparl-Radosevich et al., 1998; 1999, Fig. 2). Typically, the protein purification included protein precipitation with 40% ammonium sulfate, affinity chromatography on amylose resin (New England Biolabs), and chromatography on a mono5/5 FPLC column (Pharmacia). The difference in affinity chromatography between the enzymes is that the buffer used for affinity chromatography contained 0.2 M citrate for full-length SSIIa whereas the buffer contained 0.1M KCL for N-terminally truncated mSSIIa and <i>E.coli</i> glycogen synthase. Citrate was used to enhance binding of full-length SSIIa to the amylose affinity column. No major differences in elution profiles were observed between wild type enzymes and their mutants. The purified enzymes were electrophoresed on a 10% SDS-PAGE gel to determine enzyme purity.

**Enzyme assay and kinetic analysis**

The SS assay contained 100mM Bicine, pH 8.5, 5mM EDTA, 25mM potassium acetate, 10mM reduced glutathione, 0.5mg/ml BSA, 0.5 M citrate and either 5mg/mL amylopectin or 20mg/mL glycogen as primer except for the assay for primer kinetics. Glycogen synthase assay contains the same components as the SS assay, except that there
was no citrate in the assay mixture and only glycogen was used as primer. Reactions were initiated with the addition of [U-\(^{14}\)C]-ADPGlc into a total reaction volume of 0.1mL, and the methanol precipitation method was used to remove unreacted [U-\(^{14}\)C]-ADPGlc. The amount of enzyme was adjusted in each case so that a 4-minute or 8-minute reaction gave incorporation of [U-\(^{14}\)C]-ADPGlc in the linear range (enzyme concentration versus rate) during the time course of the assay. Kinetic parameters were analyzed with the use of a computer program (Enzfit).

Circular dichroism spectra of purified enzymes

The Iowa State University Protein Facility measured CD spectra using a J710 CD spectrometer. The spectra were taken from 190 to 260nm at room temperature with a protein concentration of 80 μg/mL in 10mM sodium phosphate, pH 8.0 in a 1mm cell.

RESULTS

Chemical modification of lysine residues in maize SSIIa

Purified maize SSIIa was chemically modified with pyridoxal 5'-phosphate, a lysine specific reagent, to investigate a possible role of lysine residue(s) in the catalysis of maize SSIIa. As shown in Fig. 3, the incubation of wild type maize SSIIa with PLP gave inactivation of the enzyme in a time and concentration dependent manner, as measured by monitoring the enzyme activity. Inactivation of SSIIa proceeded through pseudo-first-order rate kinetics, giving a linear plot of logarithmic residual activity vs. time. A double reciprocal log plot of the inactivation rate constant vs. the corresponding PLP concentration gave a straight line with a slope of 0.74, suggesting that the inactivation of mSSIIa by PLP was due to modification of at least one lysine residue (Fig. 3 insert). The substrate ADP-
glucose was found to effectively protect SSIIa against inactivation by PLP. In the presence of 2mM ADPGlc, SSIIa activity was not inactivated by 10mM PLP (Fig. 4). This suggests that lysine residue(s) may be directly involved in ADPGlc binding of maize SSIIa.

**Mutations at lysine 193 of KTGG do not affect ADP-glucose affinity of SSIIa**

To identify which lysine residue(s) may be important for the enzyme catalysis and substrate binding, site-directed mutagenesis was employed to generate single mutations of the conserved lysine residues. Since the highly conserved lysine-193 of K-T-G-G motif has been widely accepted as the putative substrate ADPG binding site of starch synthase in higher plants, this conserved lysine was mutated to arginine (R), or glutamine (Q), or glutamic acid (E). In contrast with *E.coli* glycogen synthase, the mutations at lysine-193 did not significantly change ADPG affinity of mSSIIa with either amylopectin or glycogen as a primer (Table 1). Moreover, substitution of arginine, or glutamine, or glutamic acid for lysine-193 did not result in any difference in ADP-glucose affinity of SSIIa, which is different from *E.coli* glycogen synthase. This result indicates that the ε-amino group of lysine-193 may not be involved in ADP-glucose binding of SSIIa. Therefore, clearly, lysine-193 of the conserved KTGG motif is not directly involved in ADPGlc binding of maize SSIIa.

**Mutations at lysine 193 affect enzyme catalysis of SSIIa**

The mutations at lysine-193 of the conserved K-T-G-G motif affected Vmax of SSIIa in different ways depending on which primer, amylopectin or glycogen, was used in the enzyme assay reaction (Table 1). With amylopectin as a primer, substitution of arginine or glutamine for lysine-193 decreased SSIIa enzyme activity in similar degree (about 65% decrease), whereas substitution of glutamic acid had a more negative effect on activity of
SSIIa (about a 80% decrease). The removal of a positive charge at position 193 without significant change in side chain bulkiness (K193Q and K193E) dramatically reduced $V_{\text{max}}$ of SSIIa, and K193E decreased SSIIa activity more than K193Q, suggesting that the $\varepsilon$-amino group of lysine-193 may be important for the catalytic reaction of SSIIa in the case of amylopectin as a primer. Interestingly, when glycogen was used as primer, the effects of the mutations at lysine-193 on the enzyme activity varied. Substitution of arginine for lysine-193 increased activity of SSIIa by 24%, whereas substitution of glutamine or glutamic acid decreased activity of SSIIa by 82% and 26%, respectively. In this case, neutralization of charge on the $\varepsilon$-amino group of lysine-193 (K193Q) caused the largest decrease in activity of SSIIa. One possible explanation is that neutralization of the charge on the $\varepsilon$-amino group of lysine-193 might deteriorate the glycogen affinity of SSIIa. However, our results showed that the mutation K193Q did not increase the $K_m$ for glycogen whereas K193R and K193E increased the $K_m$ for glycogen about 4-fold and 3-fold, respectively. Hence, the influence of the mutations on activity of SSIIa is related to the catalytic properties rather than binding of the primer glycogen. These results indicate that lysine-193 of the conserved “KTGG” motif may be involved in enzyme catalysis of SSIIa rather than binding of the substrate ADP-glucose.

The CD spectra of wild type maize SSIIa and mutants were measured and compared to determine whether the mutations at lysine-193 caused a major conformational change in protein (Fig. 5a). No major changes were observed in CD spectra between wild type and K193R, K193Q. The mutant K193E showed a difference in CD spectra as compared with wild type, indicating that K193E might cause a major conformational change in protein.
The functional difference in the lysine-193 between GS and SSIIa is not due to the N-terminal extension

Our research suggests a functional difference of the highly conserved lysine residue of K-T-G-G-G motif in ADP-glucose binding and enzyme catalysis between *E.coli* glycogen synthase and maize SSIIa. To verify the functional difference between maize and *E.coli*, we repeated the work of Furukawa et al. (1993). We mutated lysine-15 of *E.coli* GS to glutamine (Q) or glutamic acid (E). The *E.coli* GS wild type and its mutants K15Q and K15E were expressed in *E.coli* mutant strain EX2 (*glgA*- and *glgB*-) purified to apparent homogeneity (Fig. 1) and kinetically characterized. Table 2 summarizes the steady-state kinetic parameters of *E.coli* GS wild type and the mutants K15Q and K15E in comparison with Furukawa et al’s data (1993). Substitution of glutamic acid for lysine-15 resulted in a 24-fold increase in Km for ADP-glucose, whereas substitution of glutamine for lysine 15 caused about a 5.2-fold increase in Km for ADP-glucose of *E.coli* GS. Correspondingly, substitution of glutamine caused 7.2% decrease in Vmax of *E. coli* GS, whereas substitution of glutamic acid resulted in 95.4% decrease in Vmax of *E. coli* GS (Table 2). Basically, these results lead to the same conclusion as Furukawa’s that lysine-15 of the conserved “KTGG” motif may function as the ADP-glucose binding site of *E.coli* GS. On the other hand, we observed that neutralization of charge of the ε-amino group of lysine-15 (K15Q) resulted in a much smaller influence on ADP-glucose affinity (5-fold increase in Km for ADP-glucose) and it did not change Vmax of *E.coli* GS, which is different from Furukawa’s data (Table 2). No major changes were observed in CD spectra between wild type *E.coli* GS and its mutants (K15Q and K15E), suggesting that the differences in kinetic properties
between wild type and mutants are not caused by a global conformational change in the protein (Fig. 5b).

Structurally, the most significant difference between E.coli GS and maize SSIIa is that maize SSIIa possesses an N-terminal extension of 176 amino acids that is absent in E.coli GS (Fig. 2, Fig. 6). To examine whether the N-terminal extension is involved in the functional difference of the highly conserved lysine of “KTGG” between E.coli GS and maize SSIIa, lysine-19 of the conserved KTGG, corresponding to lysine-15 of E.coli GS and lysine-193 of the full length maize SSIIa, was mutated to glutamic acid (E) in the N-terminally truncated form of maize SSIIa. The molecular mass of the N-terminally truncated SSIIa was similar to E.coli GS (Fig. 1). Substitution of glutamic acid for lysine-19 did not result in a significant increase in Km for ADP-glucose of the N-terminally truncated maize SSIIa. When amylopectin was used as primer, Km for ADP-glucose of N-terminally truncated SSIIa and its mutant K19Q were 0.1 mM and 0.2 mM, respectively. In the case of glycogen as primer, Km for ADP-glucose of N-terminally truncated SSIIa and its mutant K19Q was 0.2 mM and 0.2 mM, respectively. This suggests that the N-terminal extension of maize SSIIa is not involved in the functional difference in ADPG binding between E.coli GS and maize SSIIa, and the C-terminus instead of the N-terminal extension is important for substrate binding, which is in agreement with Imparl-Radosevich’s (1998, 1999) results.

**DISCUSSION**

Among the major starch biosynthetic enzymes, the structure-function relationship of ADP-glucose pyrophosphorylase (AGPase) and branching enzymes has been extensively studied (Preiss and Sivak, 1996). However not much is known about the structure-function
relationship of starch synthases including granule-bound starch synthase (GBSS) and soluble starch synthases. The only hypothesis on the structure-function relationship of starch synthases in higher plants is derived from research on the enzyme mechanism of E.coli glycogen synthase. By use of affinity labeling and site-directed mutagenesis, Furukawa et al. (1990; 1993) identified lysine-15 of the conserved K-T-G-G motif as the substrate ADP-glucose binding site of E.coli glycogen synthase. E.coli glycogen synthase and plant starch synthases are functionally similar enzymes, both using ADP-glucose as substrate. Since the K-X-G-G motif is highly conserved between E.coli glycogen synthase and plant starch synthases (including all isoforms of maize starch synthase), it has been widely accepted that the lysine residue of the conserved K-X-G-G motif may also function as the ADP-glucose binding site for starch synthases in plants (Baba et al., 1993; Marshall et al., 1996; Takaoka et al., 1997; Mason-Gamer et al., 1998). Mason-Gamer et al. (1998) predicted the three-dimensional structure of E.coli glycogen synthase and GBSS by threading each of the protein sequences onto the known three-dimensional structure of mammalian glycogen phosphorylase, implying possible involvement of the conserved amino acids of GBSS and E.coli GS in active sites.

By use of chemical modification of SSIIa by pyridoxal-5'-phosphate, we have demonstrated the importance of lysine residues in starch synthase IIa. Our observation that ADP-glucose completely protected SSIIa from inactivation by PLP strongly suggests that lysine residues could be directly involved in ADP-glucose binding of maize SSIIa. Pyridoxal 5'-phosphate does not target a specific lysine residue, so there are several options for which lysine is affected. This further raises a question: which lysine residue may be responsible for ADP-glucose binding of starch synthases?
Our results strongly indicate that lysine-193 of the conserved K-T-G-G motif is unlikely to be involved in ADP-glucose binding of maize SSIIa. In contrast to *E.coli* glycogen synthase, the mutations at the conserved lysine-193 (corresponding to lysine15 of *E.coli* glycogen synthase) did not change the ADP-glucose affinity of maize SSIIa. Moreover, the ε-amino group of lysine-193 did not have the same function as that of lysine-15 in *E.coli* GS. On the other hand, the mutations at lysine-193 impose influences on enzyme activity of maize SSIIa in different ways. The effects of the mutations at lysine-193 are associated with type of primer used in enzyme reactions. When amylopectin was used as primer, the substitution of glutamic acid for lysine-193 resulted in more decrease in activity than the substitution of arginine or glutamine for lysine-193. When glycogen was used as primer, the substitution of arginine increased SSIIa activity; the substitution of glutamine resulted in a much larger decrease in activity than the substitution of glutamic acid. Clearly, lysine-193 is involved in catalysis of maize SSIIa rather than ADP-glucose binding of SSIIa, which is functionally different from *E.coli* glycogen synthase. This conclusion corrects the prevailing supposition in the starch research community that the lysine of the conserved K-T-G-G motif functions as the ADP-glucose binding site in higher plants.

Extensive characterization of conserved arginine or histidine residues shows that no conserved arginine or histidine residues are identified to be involved in ADP-glucose binding of SSIIa (Imparl-Radosevich et al., 1999). Nichols et al. (2000) characterized conserved aspartate and glutamate residues of maize SSIIb, suggesting that Asp-21 and Glu391 might be important for ADP-glucose binding of maize SSIIb. Because Asp-21 is located closely to the lysine residue of the conserved K-T-G-G on the linear sequence, Nichols et al. (2000) proposed that the carboxyl group of Asp-21 may be interacting with the adenosine ring of
ADP-glucose, and the ε-amino group of lysine of the conserved K-T-G-G may be interacting with the anionic pyrophosphate moiety of ADP-glucose. In our research, the involvement of lysine-193 of the conserved K-T-G-G in the ADP-glucose binding of maize starch synthase IIa has been ruled out. One possible scenario is that lysine-193 of the conserved K-T-G-G and aspartate-199 (corresponding to aspartate-21 of SSIIb) may be locate in the active site of SSIIa; lysine-193 is involved in catalysis of SSIIa, and the aspartate-199 residue plays a role in stabilizing ADP-glucose binding. However, based on chemical modification, lysine residues are important for the substrate ADP-glucose binding of maize SSIIa. Based on amino acid sequence alignment between E. coli GS and all isoforms of maize starch synthases, there are four highly conserved lysine residues, i.e., lysine-193 of SSIIa (corresponding to lysine-15 of E. coli GS), lysine-465 of SSIIa (lysine-273 of GS), lysine-469 of SSIIa (lysine-277 of GS), and lysine-497 of SSIIa (lysine-305 of GS). The questions are which lysine residue is involved in ADPG binding of SSIIa and whether the ADPG binding site consists of multiple lysine residues. Apart from lysine-15 of the conserved K-T-G-G motif, Furukawa et al. (1994) also characterized the function of lysine-277 (corresponding to lysine-469 of maize SSIIa) using affinity labeling and site-directed mutagenesis. They determined that lysine-277 appears unimportant for ADPG binding of E. coli GS based site-directed mutagenesis although this lysine residue was labeled by adenosine diphosphopyridoxal. Instead, lysine-277 is involved in the active site of E. coli glycogen synthase. So far, for starch synthases in plants only lysine-193 of K-T-G-G has been characterized. Further efforts to characterize other conserved lysine residues are under way to elucidate the role of lysine in the catalysis and substrate binding of maize starch synthases.
Attempting to understand the functional difference in the lysine residue of the conserved K-T-G-G, we gave attention to the most significant structural difference between E.coli glycogen synthase and maize SSIIa, that maize SSIIa possesses an N-terminal extension that is absent in E.coli glycogen synthase (Fig. 6). To understand the possible role of N-terminal extensions from different SS isoforms, we constructed chimeric enzymes to recombine the N-terminal extension and the C-terminal catalytic domain. As shown in Table 3, the replacement of SSIIa N-terminal extension by the N-terminal extension of either SSI or SSIIb dramatically decreased activity of SSIIa by about 95% to 97%, whereas the replacement of the N-terminal extension of SSIIb by the N-terminal extension of SSI or SSIIa had less decrease in activity of SSIIb (78% and 42% decrease, respectively). These results indicate that the N-terminal extension has influence on SS activity. There exists a possibility that the N-terminal extension might influence the active site by altering protein folding. Hence, there may be two possibilities for the functional difference of the conserved lysine of KTGG motif. One is that the functional difference is due to an “inherent effect” of the lysine residue; the other is that the N-terminal extension of maize SSIIa may cause a certain alteration in the enzyme structure and therefore change the function of the conserved lysine of KTGG motif. By examining the function of the lysine residue of the conserved KTGG motif in the N-terminal truncated maize SSIIa, we determined that the N-terminal extension of maize SSIIa does not influence the function of the lysine residue in ADP-glucose affinity. This result is in agreement with the previous results that the N-terminal extension is not essential to enzyme catalysis and substrate ADP-glucose binding of starch synthases (Imparl-Radosevich, et al., 1998; 1999).
It is noteworthy that substitution of arginine or glutamic acid for lysine-193 caused differences in primer preference of the enzyme as compared with wild type. Wild type SSIIa had higher enzyme activity with amylopectin as primer than with glycogen as primer, whereas the mutants K193R and K193E had higher activities with glycogen as primer than with amylopectin as primer. It was observed that there were no significant differences between Km for amylopectin and Km for glycogen of mutants K193R and K193E. It seems that primer affinity to mutant enzymes is not the major reason for the change of primer preference. Therefore, substitution of arginine or glutamic acid for lysine-193 may influence catalytic properties of SSIIa for primer glycogen and amylopectin. Hence, the change in catalytic reaction with different primers (amylopectin or glycogen) of maize SSIIa might have potential influence on amylopectin synthesis and granule biogenesis, and it deserves further characterization.

REFERENCES


Table 1. ADP-glucose and primer kinetics of maize SSIIa wild type, K193R, K193Q, K193E (mean ± standard error)

<table>
<thead>
<tr>
<th></th>
<th>Amylopectin as primer</th>
<th>Glycogen as primer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vmax</td>
<td>Km for ADPG</td>
</tr>
<tr>
<td>wild-type</td>
<td>49.98±2.87</td>
<td>0.114±0.002</td>
</tr>
<tr>
<td>K193R</td>
<td>18.19±0.64</td>
<td>0.149±0.002</td>
</tr>
<tr>
<td>K193Q</td>
<td>17.45±1.12</td>
<td>0.135±0.007</td>
</tr>
<tr>
<td>K193E</td>
<td>10.53±0.38</td>
<td>0.216±0.004</td>
</tr>
</tbody>
</table>

- Vmax values are in \( \mu \text{mol/min/mg protein} \)
- Km for ADP-glucose are expressed in mM ADP-glucose
- Km for primer are expressed as mg/ml primer
Table 2. ADP-glucose and primer kinetics of *E.coli* glycogen synthase and its lysine-15 mutants (mean ± standard error)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Vmax (glycogen)</th>
<th>Km for ADP-glucose (mM)</th>
<th>Km for glycogen (mg/ml)</th>
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<tbody>
<tr>
<td>glgA wild-type</td>
<td>Gao(1) 569.00±9.98</td>
<td>Furukawa(2) 694±29</td>
<td>Gao 0.145±0.003</td>
</tr>
<tr>
<td>glgA K15Q</td>
<td>528.19±27.48</td>
<td>120±10</td>
<td>0.760±0.049</td>
</tr>
<tr>
<td>glgA K15E</td>
<td>26.77±0.31</td>
<td>44±3</td>
<td>3.525±0.124</td>
</tr>
</tbody>
</table>

(1) Vmax are expressed in μmol/min/mg protein
(2) Kcat are expressed in s⁻¹ (Furukawa et al., 1993)
Table 3. Relative activities of the chimeric enzymes of maize starch synthases SSI, SSIIa, SSIIb (recombimants of N-terminal extension and C-terminal catalytic domain)

<table>
<thead>
<tr>
<th></th>
<th>SSIIa C-terminal catalytic domain</th>
<th>SSIIb C-terminal catalytic domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSI N-terminal extension</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>SSIIa N-terminal extension</td>
<td>100 (Wild Type)</td>
<td>58</td>
</tr>
<tr>
<td>SSIIb N-terminal extension</td>
<td>5</td>
<td>100 (Wild Type)</td>
</tr>
</tbody>
</table>
Figure 1. Amino acid sequence comparison on the conserved lysine residues between *E.coli* glycogen synthase and maize starch synthase isoforms. The sequences are obtained from following sources: *E.coli* glycogen synthase (Kumar et al., 1986), GBSS (Klosgen et al., 1986), SSI (Knight et al., 1998), SSIIa (Harn et al., 1998), SSIIb (Harn et al., 1998) and Du1 (Gao et al., 1998). The conserved lysine residues are marked with underlying lines. The lysine residues of the conserved KTGG motif are numbered as K\textsuperscript{15}, K\textsuperscript{193} and K\textsuperscript{19}, respectively, for *E.coli* glycogen synthase, full-length (FL) maize SSIIa and N-terminally truncated (TR) maize SSIIa. The corresponding mutations are labeled above or below the sequences.
Figure 2. SDS-PAGE gel electrophoresis of purified maize full-length SSIIa, N-terminally truncated SSIIa, *E. coli* glycogen synthase and their mutants. Lane 1, protein marker; Lane 2, full-length maize SSIIa; Lane 3, full-length SSIIa K193R; Lane 4, full-length SSIIa K193Q; Lane 5, full-length SSIIa K193E; Lane 6, *E. coli* glycogen synthase wild type; Lane 7, *E. coli* GS K15Q; Lane 8, *E. coli* GS K15E; Lane 9, N-terminally truncated maize SSIIa; Lane 10, N-terminally truncated SSIIa K19E.
Figure 3. Chemical modification of maize SSIIa by pyridoxal-5'-phosphate. 3μM SSIIa was incubated at 30°C with 0mM(■), 1mM(▲), 3mM(▼), 6mM(●), 10mM(★), or 15mM(□) pyridoxal-5'-phosphate in a final volume of 1ml for varying times. The reactions were terminated with addition of sodium borohydrate at fixed times, and then 5μl aliquots were withdrawn from the mixture and assayed for enzyme activity. Insert: A double reciprocal plot of reaction constants vs. ADP-glucose concentrations.
Figure 4. Protection of maize SSIIa against PLP inactivation by ADP-glucose. The enzyme SSIIa was incubated with 10mM PLP in the absence (▲) or in the presence (■) of 2mM ADPG. Neither PLP nor ADPG was added in the control (▼). At fixed times, 5µl aliquots were withdrawn from the mixture and assayed for enzyme activity.
Figure 5(a). Circular dichroism (CD) spectra of maize SSIIa wild type and its mutants K193R, K193Q, and K193E.
Figure 5 (b) Circular dichroism (CD) spectra of *E. coli* glycogen synthase wild type and their mutants K15Q and K15E.
Fig. 6. Structural comparison between E.coli glycogen synthase, full-length maize starch synthase IIa and N-terminally truncated SSIIa
Chapter 3

Involvement of the Conserved Lysine-497 in Substrate ADP-glucose Binding of Maize (Zea mays L.) Starch Synthase IIa

A paper to be submitted to Biochemistry
Zhong Gao, Peter Keeling, and Hanping Guan

ABSTRACT

We have shown that lysine-193 of the conserved K-T-G-G motif, functionally different from its counterpart in E.coli glycogen synthase, is involved in catalysis rather than ADP-glucose binding of maize (Zea mays L.) starch synthase IIa (Gao et al., 2001). To further study involvement of lysine residue(s) in catalysis and ADP-glucose binding of maize SSIIa, three conserved lysine residues—lysine-465, lysine-469, and lysine-497—were subjected to site-directed mutagenesis. Kinetic characterization showed that mutations at lysine-497 dramatically increased the Km for ADP-glucose, and moreover K497Q, K497N and K497E increased the Km for ADP-glucose more than did mutation K497H. This result strongly suggests that lysine-497 is mainly involved in substrate ADP-glucose binding, with the positive charge on the side chain at position 497 being important to ADP-glucose binding. Replacement of glutamine or glutamic acid in lysine-465 and lysine-469 resulted in complete loss of activity, whereas the conservative replacement K465R and K469R decreased activity but had no influence on ADP-glucose binding. A simulated three-dimensional protein structure model has been proposed and discussed for the possible mechanism of maize SSIIa substrate ADP-glucose binding and catalysis.
INTRODUCTION

Starch is a major storage product in higher plants, constituting the larger component of crop yield in the world and supplying an important raw material for many industrial processes. A better understanding of the mechanism of starch biosynthesis will facilitate development of a sound biotechnological strategy to improve starch yield and quality. Four major enzymes, including ADP-glucose pyrophosphorylase, starch synthase, branching enzymes, and debranching enzyme, are involved in starch biosynthesis of higher plants (Nelson and Pan, 1995; Smith et al., 1997). Starch synthase (E.C. 2.4.1.21) catalyzes the elongation of $\alpha$-(1,4) glucans by adding glucose units from ADP-glucose to the nonreducing end of the growing chain. Starch synthases play an important role in starch biosynthesis in a concerted action with branching enzymes and debranching enzyme (Jenner et al., 1993; Keeling et al., 1993; 1994).

So far, five genes coding for the starch synthase isoforms—granule bound starch synthase (GBSS), soluble starch synthases SSI, SSIIa, SSIIb, and Dull-1—have been cloned and characterized (Shure et al., 1983; Klosgen et al., 1986; Gao et al., 1998; Knight et al., 1998; Harn et al., 1998). Tightly bound to the starch granule, GBSS is mainly responsible for amylose biosynthesis. Although GBSS may have some effect on amylopectin biosynthesis, amylopectin, the major component of starch granule, is mainly elaborated by soluble starch synthases and starch branching enzymes in the soluble fraction of the amyloplast. In maize endosperm, SSI and DUI (maize SSIII) account for major contributions to soluble starch synthase activity, whereas SSIIa and SSIIb are of relatively low abundance (Cao et al., 1999). The mutation, *rug5*, in pea (*Pisum sativum* L.) SSII, the possible evolutionary counterpart of maize SSIIa, profoundly alters amylopectin structure in pea
embryos (Craig et al., 1998; Cao et al., 1999). Hence, maize SSIIa may be functionally important for starch biosynthesis and granule biogenesis.

The gene for maize SSIIa has been overexpressed in *E. coli*, and kinetically characterized (Imparl-Radosevich et al., 1999a). Current efforts have been devoted to understanding the structure-function relationship of maize SSIIa (Imparl-Radosevich et al., 1999b; Nichols et al., 2000; Gao et al., 2001). A widely accepted hypothesis concerning the catalysis of starch synthase in plants was derived from the observation that the lysine residue of the conserved K-T-G-G motif may function as the ADP-glucose binding site of *E. coli* glycogen synthase (Furukawa et al., 1990; 1993). The K-T-G-G motif is highly conserved between *E. coli* glycogen synthase and starch synthases from diverse plant species (including multiple isoforms of maize starch synthase), and *E. coli* glycogen synthase and starch synthases are functionally similar and use the same substrate, ADP-glucose. Hence, it has been suggested that the lysine residue of the conserved K-T-G-G may be involved in ADP-glucose binding of maize starch synthases (Baba et al., 1993; Marshall et al., 1996; Takaoka et al., 1997; Mason-Gamer et al., 1998). However, by use of site-directed mutagenesis we (Gao et al., 2001) have shown that the lysine-193 of the K-T-G-G motif is not directly involved in ADP-glucose binding of maize SSIIa. Instead, this lysine residue plays an important role in catalytic reaction of the enzyme.

Characterization of some conserved arginines and histidines in maize SSIIa failed to identify specific arginine or histidine residues involved in ADP-glucose binding of maize SSIIa (Imparl-Radosevich et al., 1999b). Nichols et al. (2000) observed that Asp-21 and Glu-391 might be involved in ADP-glucose binding possibly by playing a stabilizing role in substrate binding and catalysis. Further search for candidate amino acid residue(s) involved
in ADP-glucose binding will be necessary to understand the mechanisms of maize SSIIa action. Based on chemical modification lysine residue(s) may be important for ADP-glucose binding (Gao et al., 2001). Therefore, the goal of the research was to identify the possible candidate lysine residue that is directly involved in ADP-glucose binding of maize starch synthases. Moreover, a protein structure model of maize SSIIa is proposed, and the possible mechanism of catalysis also is discussed.

MATERIALS AND METHODS

Materials

Enzymes for DNA manipulation were obtained from New England Biolabs. Oligonucleotide primers for site-directed mutagenesis and DNA sequencing were synthesized and purified by the DNA sequencing facility at Iowa State University. ADP-[U-\textsuperscript{14}C] Glc was synthesized using \textsuperscript{[U-14}C]-glucose-1-phosphate (Amersham) and \textit{E.coli} ADPGlc pyrophosphorylase as described by Espada et al. (1962). All other chemicals and supplies were purchased as the highest quality available from Sigma, or as otherwise indicated.

Site-directed mutagenesis

Three conserved lysine residues other than lysine-193 of K-T-G-G were identified based on protein sequence alignment among \textit{E. coli} GS and isoforms of starch synthase (Fig. 1). Site-directed mutagenesis was carried out on maize SSIIa subcloned into the pET-21 expression vector (Novagen). A PCR in vitro, site-directed mutagenesis kit (Stratagene) was used to perform site-directed mutagenesis using the primers listed below and their reverse complements. The products of the mutagenesis reactions were transformed into
DH5α competent cells, individually, followed by DNA amplification and sequencing to confirm the presence of the desired mutations. To ensure that there was no undesired mutation introduced by PCR, the DNA fragment containing the desired mutation was re-subcloned into the wild type plasmids. The entire re-subcloned DNA fragments were then sequenced again.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>K465R</td>
<td>ExS 119</td>
<td>5'-ACA CTC GAC GCT GGA AGG CGG CAG TGC AA-3'</td>
</tr>
<tr>
<td>K465Q</td>
<td>ExS 121</td>
<td>5'-ACA CTC GAC GCT GGA CAG CGG CAG TGC AA-3'</td>
</tr>
<tr>
<td>K469R</td>
<td>ExS 123</td>
<td>5'-A AAG CGG CAG TGC AGG GCC GCC CTG CA-3'</td>
</tr>
<tr>
<td>K469Q</td>
<td>ExS 125</td>
<td>5'-A AAG CGG CAG TGC CAG GCC GCC CTG CA-3'</td>
</tr>
<tr>
<td>K469E</td>
<td>ExS 167</td>
<td>5'-A AAG CGG CAG TGC GAG GCC GCC CTG CA-3'</td>
</tr>
<tr>
<td>K497R</td>
<td>ExS 127</td>
<td>5'-T CTG GAT GGA CAG AGG GGC GTG GAC ATC ATC GG-3'</td>
</tr>
<tr>
<td>K497H</td>
<td>ExS 302</td>
<td>5'-CGT CTG GAT GGA CAG CAC GCC GTG GAC ATC ATC GG-3'</td>
</tr>
<tr>
<td>K497Q</td>
<td>ExS 129</td>
<td>5'-T CTG GAT GGA CAG CAG GCC GTG GAC ATC ATC GG-3'</td>
</tr>
<tr>
<td>K497N</td>
<td>ExS 287</td>
<td>5'-CTG GAT GGA CAG AAC GCC GTG GAC ATC ATC GG-3'</td>
</tr>
<tr>
<td>K497E</td>
<td>ExS 289</td>
<td>5'-CTG GAT GGA CAG GAG GCC GTG GAC ATC ATC GG-3'</td>
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<td>K465R/K469R</td>
<td>ExS 191</td>
<td>5'-GAC GCT GGA AGG CGG CAG TGC AGG GCC GCC CTG-3'</td>
</tr>
</tbody>
</table>

The double mutation SSIIa K465R/K469R was generated using PCR site-directed mutagenesis with primer ExS 191 and its reverse complement. To generate other double mutations, K193R/K465R, K193R/K469R, K193R/K497Q and K193Q/K497Q, the EcoRI-NcoI fragment carrying the desired mutation at lysine-465, lysine-469, or lysine-497 from pET21a-SSIIa was cloned into the EcoRI-NcoI cloning site of pET21a-SSIIa carrying a mutation at lysine-193. Sequence analysis of the recombinant maize SSIIa verified that the desired mutation had been generated and that no other mutation was present.
Expression and purification of the mutant enzymes

SSIIa and its mutant plasmids were individually transformed into *E. coli* mutant strain EX2 [glgA- and glgB-] which allows the measurement of activities of mutant enzymes in the absence of a bacterial glycogen synthase and branching enzyme. Overnight cultures were diluted 1:20 into 10 L of LB broth and grown at 37 °C until OD600 reached 0.6. Expression of SS enzymes was induced by the addition of 1 mM IPTG. The cultures were then grown at room temperature for 4 hours. The cell pellet was frozen in nitrogen liquid and stored at -70°C after harvesting by centrifugation at 9600g for 10 minutes. Western blotting showed that all of mutants were expressed in a similar level to wild type in *E. coli* (data not shown).

Maize SSIIa and its mutants were purified to apparent homogeneity with a procedure similar to that described previously (Imparl-Radosevich et al., 1999a). Typically, the protein purification included protein precipitation with 40% ammonium sulfate, affinity chromatography on amylose resin, and chromatography on a mono5/5 FPLC column (Pharmacia). No major differences in elution profiles were observed between wild type enzymes and their mutants. The purified enzymes were electrophoresed on a 10% SDS-PAGE gel to determine the enzyme purity.

Enzyme assay and kinetic analysis

The SS assay contained 100mM Bicine (pH 8.5), 5mM EDTA, 25mM potassium acetate, 10mM reduced glutathione, 0.5mg/ml BSA, 0.5M citrate and either 5mg/mL amylopectin or 20mg/mL glycogen as primer. The glycogen synthase assay contained the same components as the SS assay except that there was no citrate in the assay mixture and only glycogen was used as primer. Reactions were initiated with the addition of [U-14C]-ADPGlc (225 μCi / mmol) into a total reaction volume of 0.1mL, and the methanol
precipitation method was used to remove un-reacted [U-\(^{14}\)C]-ADPGlc. The amount of enzyme was adjusted in each case so that the reaction gave incorporation of [U-\(^{14}\)C]-ADPGlc in the linear range (enzyme concentration versus rate) during the time course of the assay. Kinetic parameters were analyzed with the use of a computer program (Enzfit).

**Circular dichroism spectra of purified enzymes**

The CD spectra were measured by the Iowa State University Protein Facility using a J710 CD spectrometer. The spectra were taken from 190 to 260nm at room temperature with a protein concentration of 80 \(\mu\)g/mL in 10mM sodium phosphate, pH 8.0, in a 1mm cell.

**Generation of the three dimensional structure model of maize SSIIa**

Because no protein sequence of known three-dimensional structure is homologous to maize starch synthase IIa with 25% or more similarity, homology modeling cannot be used for modeling the 3D structure of maize SSIIa (Peitsch, 1996). However, the protein folding recognition technique (3D-pssm) has been applied successfully to modeling the 3D structure of SSIIa (Kelley et al., 2000). We submitted the protein sequence of maize SSIIa to the automated 3D-pssm server (http://www.bmm.icnet.uk/~3dspssm) for structure modeling. Each model was analyzed individually. The best-fit model with the highest confidence was used for analysis.

**RESULTS**

**Mutations at lysine-465 and lysine-469**

The effect of mutations at lysine-465 and lysine-469 on the SSIIa activities was similar. It seems that substitution of glutamine for lysine-465 and lysine-469 deteriorated the SSIIa activities much more than substitution of arginine for lysine-465 or lysine-469.
Mutants K465R and K469R decreased SSIIa activity by 47% to 68%; whereas K465Q and K469Q were not active. Because lysine-227 of E.coli glycogen synthase, corresponding to lysine-469 of maize SSIIa, has been shown to be involved in the active site of E. coli glycogen synthase (Furukawa et al., 1994), we wanted to characterize further the two closely-located lysine residues, lysine-465 and lysine-469. We purified and characterized K465R and K469R. However, our attempt to purify K465Q, K469Q, and K469E failed because the activities of these three mutants were very low or not detectable using either amylopectin or glycogen as primer.

Kinetic parameters for SSIIa and the mutants K465R and K469R were measured using either amylopectin or glycogen as primers in the presence of 0.5 M citrate (Table 1). Mutant K465R showed 53% and 47% decreases in Vmax with amylopectin and glycogen as primer; K469R showed 68% and 59% decreases in Vmax for amylopectin and glycogen as primer. However, kinetic characterization of K465R and K469R did not provide evidence for their involvement in ADP-glucose binding of SSIIa. Substitution of arginine for lysine-465 and lysine-469 did not change the Km for ADP-glucose dramatically as compared with wild type (Table 1). Both K465R and K469R showed a three-fold increase in apparent Km for amylopectin and 1.5- and 2.6-fold increase in apparent Km for glycogen. Moreover, using amylopectin as primer, double mutant K465R/K469R resulted in a 98% decrease in SSIIa enzyme activity (data not shown). These results indicate that lysine-465 and lysine-469 are important for enzyme catalysis of SSIIa. However, these results cannot provide enough evidence to rule out the possibility of involvement of lysine-465 or lysine-469 in ADP-glucose binding. The fact that mutations K465Q and K469Q (or K469E) led to almost
complete loss of activity of SSIIa suggests that lysine-465, lysine-469 and their ε-amino
group may be very important for enzyme activity of SSIIa.

**Mutations at lysine-497**

Different mutations at lysine-497 had complex effects on enzyme activity. Using
amylopectin as primer, the mutant K497R showed no activity; K497H decreased SSIIa
activities much more than K497Q, K497N and K497E (Table 2). This implies that the size
of side chain might influence catalysis of maize SSIIa although the substitution of arginine or
histidine for lysine-497 was thought to be conservative based on charge of side chain. We
purified K497H, K497Q, K497N, and K497E to apparent homogeneity and kinetically
characterized them; K497R was totally inactive and could not be purified.

Mutants K497H, K497Q, K497N, and K497E were kinetically characterized using
either amylopectin or glycogen as primer in the presence of 0.5 M citrate (Table 2). Substitution of histidine (H) for lysine-497 dramatically decreased Vmax of SSIIa by 99%
and 95%, respectively, for amylopectin and glycogen as primer. It is also noteworthy that
substitution of arginine (R) for lysine-497 resulted in complete loss of activity. These results
suggest that size of the side chain at this amino acid position may be very important for
enzyme catalysis. Substitution of glutamine or asparagine for lysine-497 decreased Vmax of
SSIIa by 73% and 77%, respectively, in the case of amylopectin as primer, whereas the same
mutations decreased activity of SSIIa by only 30% and 23%, respectively, in the case of
glycogen as primer (Table 2). Mutations K497Q and K497N decreased SSIIa activities in a
similar degree, suggesting that length of side chain may have no important impact on
catalysis when the side chain is linear or when length of the side chain is less than that of
lysine. However, when glutamine (Q) at position 497 was replaced by its acidic counterpart
glutamic acid (E), Vmax decreased by 68% and 76%, respectively, for amylopectin and glycogen as primer. These results suggest that charge of the side chain at position 497 might play an important role in catalysis of SSIIa. Interestingly, all active mutants of lysine-497 had higher Vmax with glycogen as primer than with amylopectin as primer, which is different from wild type. Moreover, Km for amylopectin of each lysine-497 mutant was lower than, or similar to, the Km for glycogen of the corresponding mutant (Table 3). Hence, these mutations at lysine-497 may affect the catalytic reaction of SSIIa with the primer.

Mutations at lysine-497 had dramatic influence on substrate ADP-glucose binding of SSIIa (Table 2). The degree of influence varied depending on mutations. In the case of amylopectin as primer, substitution of histidine for lysine-497 resulted in an 11-fold increase in Km for ADP-glucose, whereas mutations K497Q, K497N, and K497E showed 31- to 49-fold increases in Km for ADP-glucose. In the case of glycogen as primer, mutation K497H showed a 26-fold increase in Km for ADP-glucose, whereas K497Q, K497N, and K497E caused 54- to 70-fold increases in Km for ADP-glucose (Table 3, Figure 2). Mutation K497H deteriorated SSIIa activities much more than K497Q, K497N and K497E, whereas K497H had a much less negative impact on ADP-glucose affinity than the others. This implies that substitution of histidine for lysine-497 mainly influenced SSIIa catalysis; on the other hand, K497Q, K497N and K497E mainly had an impact on ADP-glucose binding. These results suggest that lysine-497 plays an important role in ADP-glucose binding of maize SSIIa, and a positive charge of the side chain at this position may be important for binding with ADP-glucose.

It is noteworthy that the Km for ADP-glucose of lysine-497 mutants was higher with glycogen as primer than with amylopectin as primer (Table 2). This result implies that
mutants at lysine-497 influenced the interaction between ADP-glucose and primer, and that
type of primer, amylopectin vs. glycogen, affected ADP-glucose affinity of the mutants.
There may be two possibilities with respect to the interaction between primer and ADP-
glucose binding. One is that there may be a correlation between primer affinity and ADP-
glucose affinity because mutations at lysine-497 also exhibited about 5- to 11-fold increases
in the Km for amylopectin and about 14- to 27-fold increases in the Km for glycogen (Table
3). The other is that differences in the interaction between the mutant enzymes and the
different primers may have an impact on ADP-glucose binding. Correlation analysis showed
that there was no significant correlation between ADP-glucose affinity and primer affinity.
The correlation coefficient between Km for ADP-glucose and Km for amylopectin was 0.37,
and the correlation coefficient between Km for ADP-glucose and Km for glycogen was 0.46.
Hence, it is likely that the primer difference in ADP-glucose binding of the mutant enzymes
was due to differences in the catalytic reaction with the primers.

**Circular dichroism (CD) spectra of purified SSIIa mutants**

The CD spectra of purified SSIIa K465R and K469R could not be measured because
protein concentrations of these purified enzymes were lower than the concentration required
for CD spectra measurement. For mutants K497H, K497Q, K497N, and K497E, no major
changes were observed in the CD spectra between wild type SSIIa and the mutants (Fig. 3).
This indicates that local effects of the mutations rather than a global conformation change
caused changes in kinetic properties.

**Three-dimensional structure model of maize SSIIa generated by folding recognition**

To further study the structure-function relationship of maize starch synthases, a
protein fold recognition technique (3D-pssm) was applied to predict 3D protein structure
models. The protein sequences of maize SSI, SSIIa, SSIIb, GBSS, and C-terminal catalytic domain of DUI (SSIII) were individually threaded onto known three dimensional protein structures (Kelley et al., 2000). We chose potential models with the highest confidence (more than 95% certainty) out of a total of 20 generated models. For maize SSI, SSIIa, SSIIb, GBSS and the C-terminal catalytic domain of DUI, the best fit was to UDP-N-acetylglucosamine 2-epimerase (isomerase) from *E. coli*. The second best fit for SSI, SSIIa, and SSIIb was to beta-glucosyltransferase and glycogen phosphorylase, with lower confidence level (70% to 95% certainty); the second best fit for the C-terminal catalytic domain of DUI was to *E. coli* MurG (a membrane-associated glycosyltransferase). UDP-N-acetylglucosamine 2-epimerase has structural homology with glycogen phosphorylase and T4 phage beta-glucosyltransferase (Campbell et al., 2000). The protein structure modeling demonstrated that SSI, SSIIa, SSIIb, GBSS, and the C-terminal catalytic domain might have similar protein structure. The structural alignment showed that lysine-193 of SSIIa was close to lysine-15 of UDP-N-acetylglucosamine 2-epimerase (10 amino acids apart), asp-199 of SSIIa was close to Glu-12 of UDP-N-acetylglucosamine 2-epimerase (1 amino acid apart), and lys-497 of SSIIa was close to His-213 of UDP-N-acetylglucosamine 2-epimerase (8 amino acids apart). Comparing the three dimensional structure model of SSIIa with the 3D structure of UDP-N-acetylglucosamine 2-epimerase, it was observed that lysine-193, aspartic acid 199, and lysine-497 were threaded to the active site of UDP-N-acetylglucosamine 2-epimerase. In the 3D structure model, lysine-193, aspartic acid 199, and lysine-497 were located in a deep cleft corresponding to the active site of UDP-N-acetylglucosamine 2-epimerase, each of these residues corresponding to lysine-15, glutamic acid 296, and histidine 213 (Fig. 4). No structural and functional counterpart was identified in the 3D
structure of UDP-N-acetylglucosamine 2-epimerase for lysine-465 or lysine-469 of maize SSIIa.

**DISCUSSION**

Characterizing the structure-function relationship of maize starch synthase will greatly improve our understanding of its catalytic mechanism. Based on chemical modification experiments, lysine, arginine, aspartate and glutamate have been shown to be important for the substrate ADP-glucose binding of maize starch synthases (Gao et al., 2001; Imparl-Radosevich, 1999b; Nichols et al., 2000). The conserved Asp-21 and Glu-391 have been demonstrated to be important for ADP-glucose binding of the truncated maize SSIIb, possibly by playing a stabilizing role in ADP-glucose binding and catalysis (Nichols et al., 2000). It has been expected that lysine, or arginine, of histidine may be involved in ADP-glucose binding of SS. However, no conserved arginine or histidine residue was found to be involved in substrate ADP-glucose binding based on site-directed mutagenesis (Imparl-Radosevich et al., 1999b). Furthermore, the widely accepted putative ADP-glucose binding site of plant starch synthases, the lysine residue of the highly conserved K-T-G-G motif, has been ruled out (Gao et al., 2001). To identify the potential ADP-glucose binding site, we further characterized other lysine residues lysine-465, lysine-469 and lysine-497 that are highly conserved among *E. coli* glycogen synthase and starch synthase isoforms. Kinetic characterization of the purified mutants SSII K465R and K469R showed that substitution of arginine for lysine-465 or lysine-469 did not change ADP-glucose affinity. However, the possibility of involvement of lysine-465 and lysine-469 in ADP-glucose binding cannot be ruled out because substitution of glutamine or glutamic acid resulted in complete loss of
enzyme activity of SSIIa. Lysine-277 of *E. coli* glycogen synthase (corresponding to lysine-469 of SSIIa) has been shown to be involved in catalysis instead of ADP-glucose binding (Furukawa et al., 1994). When lysine-277 was mutated to glutamine, the activity of glycogen synthase decreased by 90% (Furukawa et al., 1994). For SSIIa, K469R decreased SSIIa activities by 59% to 68%, whereas K469Q totally inactivated SSIIa. Hence, lysine-469 may be important for SSIIa catalysis.

Our research strongly suggests that lysine-497 is directly involved in ADP-glucose binding of maize SSIIa. It is worth noting that the replacement of lysine with glutamine (K497Q), asparagine (K497N), or glutamic acid (K497E) had much less effect on Vmax than the replacement of lysine with arginine (K497R) or histidine (K497H). However, the mutations K497Q, K497N, and K497E increased the Km for ADP-glucose 31- to 49-fold and 54- to 73-fold, respectively, for amylopectin as primer and for glycogen as primer. This suggests that lysine-497 may be directly involved in ADP-glucose binding. Replacement of lysine-497 with histidine resulted in almost complete loss of activity (more than 99% decrease of activity), whereas this replacement caused much less increase in the Km for ADP-glucose than other mutations (K497Q, K497N, and K497E). This suggests that the positive charge of lysine-497 is important for substrate ADP-glucose binding, and size restriction operates on enzyme catalysis at amino acid position 497. Moreover, the effects of these mutations at lysine-497 are not caused by global conformational changes detectable in CD spectra data. Hence, we conclude that the conserved lysine-497 is directly involved in ADP-glucose binding of maize SSIIa.

A three-dimensional structure of an enzyme is important for assigning a role to a specific amino acid residue, and for understanding the structure-function relationships of an
enzyme. So far, no three-dimensional protein structure of starch synthases or glycogen synthase that is based on experimental data has been published. Mason-Gamer et al. (1998) threaded *E. coli* glycogen synthase (glgA) and granule bound starch synthase (GBSS) onto the known three-dimensional protein structure of mammalian glycogen phosphorylase. In terms of their threading results, the functionally important residues lysine-15 and lysine-277 of *E. coli* glycogen synthase are located at each end of the active site of glycogen phosphorylase. These findings match the experimental result that lysine-15 is involved in substrate ADP-glucose binding, and lysine-277 participates in catalysis of *E. coli* glycogen synthase (Furukawa et al., 1990; 1993; 1994). Because these lysine residues are highly conserved between *E. coli* glycogen synthase and plant starch synthases, it has been hypothesized that the corresponding lysine residues are also important for substrate binding and catalysis. In our protein threading results, maize starch synthase IIa fits to UDP-N-acetylglucosamine 2-epimerase from *E. coli* with the highest confidence. Moreover, maize SSIIa fits to beta-glucosyltransferase and glycogen phosphorylase with the second highest degree of confidence, which is consistent with the fact that UDP-N-acetylglucosamine 2-epimerase shows homologous structure with beta-glucosyltransferase and glycogen phosphorylase (Campbell et al., 2000). In this model, lysine-193 (Gao et al., 2001), lysine-497, and aspartic acid 199 (Nichols et al., 2000) of maize SSIIa geometrically correspond to lysine-15, histidine-213, and glutamic acid 296 of UDP-N-acetylglucosamine 2-epimerase, respectively (Fig. 4). Lysine-15, histidine-213, and glutamic acid 296 are located in the active site of UDP-N-acetylglucosamine 2-epimerase with important function (Fig. 4). Glu-296 has been confidently assigned a role in binding the UDP portion of the substrate. Lysine-15 and histidine-213 are found in the active site, close to where GlcNAc portion of
UDP-GlcNAc is expected to bind. The role of these residues well matches the observed function of conserved lysine-193, aspartate-199 (corresponding to aspartate-21 of truncated SSIIb), and lysine-497 in maize SSIIa (Nichols et al., 2000; Gao et al., 2001). Other maize starch synthase isoforms, including SSI, SSIIb, GBSS, and the C-terminal catalytic domain of DU1 (SSIII) have shown a similar 3D structure model based on threading, suggesting the potentiality of mapping the structure-function relationships of SSIIa to other SS isoforms.

A possible scenario of the structure-function relationships in maize SSIIa is that lysine-193 is involved in catalysis, and lysine-497 and aspartate-199 participate in substrate ADP-glucose binding of SSIIa (Fig. 4). Although lysine-497 and aspartate-199 are located far apart in the linear protein sequence, the two key amino acids may be closely located in a geometrical sense. The ε-amino group of lysine-497 may directly interact with the anionic pyrophosphate moiety of ADP-glucose; the carboxyl group of aspartate-199 may play a stabilizing role by interacting with the adenosine ring of ADP-glucose.

Our results have demonstrated functional differences in conserved lysine residues between *E. coli* glycogen synthase and maize starch synthase (Gao et al., 2001, Table 4). In *E. coli* glycogen synthase, lysine-15 is involved in substrate ADP-glucose binding, and lysine-277 is important for enzyme catalysis (Furukawa et al., 1990; 1993; 1994). However, the characterization of maize SSIIa illustrates a different scenario of function for the conserved lysine residues in plants. Lysine-193 of K-T-G-G motif (corresponding to lysine-15 of *E. coli* glycogen synthase) is involved in catalysis rather than ADP-glucose binding (Gao et al., 2001). Instead, lysine-497 (corresponding to lysine-305 of *E. coli* GS) is directly involved in substrate ADP-glucose binding. Substitution of glutamine or glutamate for lysine-465 and lysine-469 completely inactivated SSIIa activities, suggesting that these two
lysine residues may be important for catalysis, which is similar to lysine-277 of *E. coli* glycogen synthase. The 3D protein structure model shows that these two lysine residues are not close to the deep cleft where the active site may be located. Hence, the roles of these two conserved lysine residues are not certain. For *E. coli* glycogen synthase, further efforts to characterize the conserved lysine-305 (corresponding to lysine-497 of SSIIa) will possibly elucidate the role of the conserved lysine in the catalysis and substrate binding of *E. coli* GS.

Our previous results (Gao et al., 2001) show unequivocally that lysine193 of the conserved KTGG is not directly involved in ADPG binding of SSIIa, instead, lysine497 may function as the ADPG binding site of SSIIa. This finding will lead to a better understanding of the molecular mechanisms of ADPG binding of starch synthases, and facilitate genetic engineering efforts to improve catalytic efficiency of SS. It has been suggested that alterations in the supply of ADP-glucose, the substrate of starch synthases, can affect the production of amylose, the branching of amylopectin and the size of granules (Van den Koornhuyse et al., 1996; Lloyd et al., 1999). Interestingly, this phenomenon is attributed to the fact that the granule-bound starch synthase (GBSS) has a lower ADPG affinity than soluble starch synthases (MacDonald et al., 1985), and therefore GBSS may be much more greatly affected by a reduction in ADPG concentration than the soluble starch synthases (such as SSIIa). Hence, to enhance ADPG affinity of GBSS would bring beneficial changes to starch quantity and quality, particularly to the relative proportion of unbranched amylose to branched amylopectin. To this end, the elucidation of mechanisms of ADPG binding in the presented research may provide valuable information for further efforts to improve starch biosynthesis.
REFERENCES


Table 1. ADP-glucose and primer kinetics of maize SSIIa wild type, K465R, and K469R (mean ± standard error)

<table>
<thead>
<tr>
<th></th>
<th>Amylopectin as primer</th>
<th>Glycogen as primer</th>
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<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$ for ADPG</td>
</tr>
<tr>
<td>Wild-type</td>
<td>49.98±2.87</td>
<td>0.114±0.002</td>
</tr>
<tr>
<td>K465R</td>
<td>23.53±1.92</td>
<td>0.232±0.046</td>
</tr>
<tr>
<td>K469R</td>
<td>15.78±0.18</td>
<td>0.371±0.105</td>
</tr>
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</table>
Table 2. $V_{\text{max}}$ and $K_m$ for ADP-glucose of maize SSIIa wild type, K497H, K497Q, K497N, and K497E (mean ± standard error)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$ for ADPG</td>
</tr>
<tr>
<td>Wild-type</td>
<td>49.98±2.87</td>
<td>0.114±0.002</td>
</tr>
<tr>
<td>K497H</td>
<td>0.24±0.02</td>
<td>1.285±0.173</td>
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<tr>
<td>K497Q</td>
<td>13.52±0.14</td>
<td>5.623±0.162</td>
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<tr>
<td>K497N</td>
<td>11.56±0.84</td>
<td>3.577±0.311</td>
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<tr>
<td>K469E</td>
<td>4.38±0.25</td>
<td>4.612±0.275</td>
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Table 3. V<sub>max</sub> and primer kinetics of maize SSIIa wild type, K497H, K497Q, K497N, and K497E (mean ± standard error)

<table>
<thead>
<tr>
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<th>Amylopectin as primer</th>
<th>Glycogen as primer</th>
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<tr>
<td></td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; for</td>
</tr>
<tr>
<td></td>
<td></td>
<td>amylopectin</td>
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<tr>
<td>Wild-type</td>
<td>47.63±2.04</td>
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<td>K497H</td>
<td>0.26±0.02</td>
<td>2.154±0.434</td>
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<tr>
<td>K497Q</td>
<td>13.52±0.14</td>
<td>1.266±0.119</td>
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<tr>
<td>K497N</td>
<td>9.44±0.83</td>
<td>0.920±0.036</td>
</tr>
<tr>
<td>K469E</td>
<td>4.09±0.27</td>
<td>2.332±0.432</td>
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Table 4. *E. coli* glycogen synthase vs. maize starch synthase IIa: Summarization of functional differences of the conserved lysine residues

<table>
<thead>
<tr>
<th>Lysine Residues</th>
<th>E.coli Glycogen Synthase</th>
<th>Maize Starch Synthase</th>
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</thead>
<tbody>
<tr>
<td>&quot;KTGG&quot;</td>
<td>Mutations at lysine-15 affect Km for ADP-glucose significantly—ADPG binding site of E.coli glycogen synthase (Furukawa 1993; Gao repeat, 2001)</td>
<td>Lysine-193 is not directly involved into ADP-glucose binding, but participates in catalysis of SSIIa (Gao et al., 2001)</td>
</tr>
<tr>
<td>K15 (<em>E. coli</em> GS) K193 (maize SSIIa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K273 (<em>E. coli</em> GS) K465 (maize SSIIa)</td>
<td>UNKNOWN</td>
<td>SSIIa cannot tolerate substitution of glutamine or glutamic acid for lysine-465. K465R decreased activity, but had no influence on ADP-glucose binding</td>
</tr>
<tr>
<td>K277 (<em>E. coli</em> GS) K469 (maize SSIIa)</td>
<td>Mutation at lysine-277 does not affect Km for ADPG, but it affected Vmax dramatically—may be important for active site rather than substrate binding site (Furukawa 1994)</td>
<td>Similar to lysine-465, but K469R decreased SSIIa activity more than K465R.</td>
</tr>
<tr>
<td>K305 (<em>E. coli</em> GS) K497 (maize SSIIa)</td>
<td>UNKNOWN</td>
<td>Mutations at lysine-497 dramatically increased Km for ADPG (40- to 50-fold)—lysine-497 is directly involved in ADP binding site.</td>
</tr>
</tbody>
</table>
Figure 1. Amino acid sequence comparison of the conserved lysine residues between *E. coli* glycogen synthase and maize starch synthase isoforms. The sequences were obtained from the following sources: *E. coli* glycogen synthase (Kumar et al., 1986), GBSS (Klosgen et al., 1986), SSI (Knight et al., 1998), SSIIa (Ham et al., 1998), SSIIb (Ham et al., 1998) and Du I (Gao et al., 1998). The conserved lysine residues are marked with underlines. The conserved lysine residues of *E. coli* glycogen synthase and maize starch synthase are numbered. The corresponding mutations are labeled below the sequences.
Figure 2. ADP-glucose kinetics of SSIIa K497H and K497Q. ADP-glucose kinetics was determined in the presence of saturating conditions of primer, 20 mg/ml with glycogen as primer and 5 mg/ml with amylopectin as primer. Figure (A) shows ADP-glucose curves for SSIIa K497H (•*•), plotted on the left Y-axis, and SSIIa K497Q (H), plotted on the right Y-axis, with amylopectin as primer. Figure (B) shows ADP-glucose curves for SSIIa K497H (•*•), plotted on the left Y-axis, and SSIIa K497Q (■), plotted on the right Y-axis, with glycogen as primer.
Figure 3. Circular Dichroism (CD) spectra of maize SSIIa and its mutants at the conserved lysine-497 (K497H, K497Q, K497N, and K497E)
Figure 4. Three-dimensional protein structure modeling of maize SSIIa. Figure (A) shows the three-dimensional protein structure of UDP-N-Acetylglucosamine 2-epimerase (Campbell et al., 2000). Three functionally important amino acid residues: lysine-15, histidine-213, and glutamic acid 296 are labeled. Figure (B) shows the three-dimensional structure model of maize SSIIa generated (confidence level ≥ 95%) by the 3D-pssm automated fold recognition technique (Kelley et al., 2000). Six amino acid residues are labeled: lysine-193, lysine-497, aspartate-199 (Nichols et al., 2000), lysine-465, lysine-469, and arginine-214 (Imparl-Radosevich et al., 1999). A deep cleft is visible on the lower part, which is probably the active site.
Chapter 4.

Citrate influences the catalytic chain elongation specificity of maize starch synthase IIa

A paper to be submitted to Archives of Biochemistry and Biophysics

Zhong Gao, Peter Keeling, and Han-Ping Guan

ABSTRACT

This study investigated the influence of citrate on the catalytic chain elongation specificities of maize (Zea mays L.) soluble starch synthase SSIIa. SSIIa, an enzyme involved in amylopectin biosynthesis, and its mutants K193R, K193Q, and K497Q had higher activities with glycogen than with amylopectin in absence of citrate, suggesting that SSII and its mutants preferentially elongate shorter outer chains in absence of citrate. However, the catalytic chain elongation specificities of SSIIa and its mutants differed in response to citrate concentration. With increase of citrate concentration, wild type SSIIa showed a property that the catalytic chain elongation specificity of the enzyme could be switched from glycogen to amylopectin. The catalytic chain elongation specificity of K193Q was more sensitive than wild type to the influence of citrate; switching to amylopectin more sharply. However, the catalytic chain elongation specificities of mutants K193R and K497Q was insensitive to citrate, losing the switching property of wild type. Citrate was involved in the interaction between enzyme and primer rather than the interaction between ADP-glucose and enzyme. These results suggest that in vitro citrate can affect catalytic specificity of SSIIa in chain elongation specificity through regulating primer binding to the enzyme.
INTRODUCTION

Starch synthase catalyzes the elongation of α-(1,4) glucans by adding glucose units from ADP-glucose to the nonreducing end of the growing chain. GBSS is considered to be mainly responsible for amylose biosynthesis although some evidence supports the notion that GBSS is also involved in amylopectin biosynthesis (Tsai, 1974; Shure et al., 1983; Smith et al., 1997). Soluble starch synthases are believed to act mainly on amylopectin biosynthesis around the starch granule surface. Boyer and Preiss (1981) found that there were two major forms of soluble starch synthase in maize endosperm, SS activity peak I and SS activity peak II (sometimes referred to as SS Type I or SS Type II), based on the elution profile from anion exchange columns. The major differences between SS activity peak I and peak II is that the maximal velocity of Type I SS is greater with rabbit (Oryctolagus cuniculus) liver glycogen than with amylopectin, and Type II SS is less active with glycogen than with amylopectin (Sivak and Preiss, 1998). This suggests that Type I SS may preferentially elongate short exterior chains that are more prevalent in glycogen than in amylopectin, whereas Type II SS may preferentially elongate longer outer chains prevalent in amylopectin.

So far, the genes for four maize soluble starch synthase enzymes have been identified, coding for SSI, SSIIa, SSIIb, and Dul (Klosgen et al., 1986; Gao et al., 1998; Knight et al., 1998; Harn et al., 1998). SSI corresponds to SS activity peak I, preferentially elongating shorter outer chains regardless of the presence of citrate (Imparl-Radosevich et al., 1998). Moreover, SSI has a greater affinity for longer outer chains than for shorter outer chains, whereas SSI enzyme has a higher activity with shorter outer chains than with longer outer chains (Commuri and Keeling, 2001). Commuri and Keeling (2001) suggested that SSI enzyme catalytically prefers shorter outer chains, but stronger binding of SSI to longer outer
chains may render the enzyme catalytically incapable. Characterization of the mutant *dull1* and *sugary2* suggests that soluble starch synthases other than SSI may be involved in synthesizing intermediate chains (Wang et al., 1993; Takeda et al., 1993; Gao et al., 1998; Harn et al., 1998). SSIII (Du1) has been proposed to be responsible for SS activity peak II based on molecular mass and transcription pattern (Gao et al., 1998; Cao et al., 1999). However, the enzyme property of SSIII is not known because so far SSIII has not been purified and characterized. Apart from the observation that maize SSIIa has a higher activity with amylopectin than with glycogen in the presence of 500 mM citrate (Imparl-Radosevich et al., 1999), little is known about the interaction between SSIIa and primers with different outer chain length. Hence, further characterization of the interaction between SSIIa and primers will facilitate a better understanding of catalysis of SSIIa, and of the potential role of SSIIa in starch biosynthesis.

Citrate can stimulate starch synthase activity (Sivak and Preiss, 1998), and also can increase primer affinity and stabilize starch synthase at high temperature (Imparl-Radosevich et al., 1998; 1999). However, the role of citrate in regulation of starch synthases is still not understood. This study investigates the influence of citrate on the catalytic specificity of maize SSIIa in chain elongation. Several site-directed mutants were found to change the catalytic chain length elongation specificity of SSIIa wild type. The potential role of citrate in regulation of catalysis of SSIIa is discussed. This work will provide important biochemical clues to manipulate chain-length preferences of SSIIa, and potentially to modify starch structure.
MATERIALS AND METHODS

Materials

Enzymes for DNA manipulation were obtained from New England Biolabs. Oligonucleotide primers for site-directed mutagenesis and DNA sequencing were synthesized and purified by the DNA sequencing facility at Iowa State University; ADP-[U-\(^{14}\)C]-Glc was synthesized using [U-\(^{14}\)C]-glucose-1-phosphate (Amersham) and \textit{E.coli} ADPGlc pyrophosphorylase as described (Espada et al., 1962); all other chemicals were purchased as the highest quality available from Sigma, or as otherwise indicated.

Site-directed mutagenesis

Site-directed mutagenesis was carried out on full-length maize SSIIa as described in Chapter 2 and Chapter 3. The primers used for site-directed mutagenesis are listed below.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K465R</td>
<td>ExS119</td>
<td>5'-ACA CTC GAC GCT GGA AGG CGG CAG TGC AA-3'</td>
</tr>
<tr>
<td>K465Q</td>
<td>ExS121</td>
<td>5'-ACA CTC GAC GCT GGA CAG CGG CAG TGC AA-3'</td>
</tr>
<tr>
<td>K469R</td>
<td>ExS123</td>
<td>5'-A AAG CGG CAG TGC AGG GCG GCC CTG CA-3'</td>
</tr>
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<td>K469Q</td>
<td>ExS125</td>
<td>5'-A AAG CGG CAG TGC CAG GCG GCC CTG CA-3'</td>
</tr>
<tr>
<td>K469E</td>
<td>ExS167</td>
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</tr>
<tr>
<td>K497R</td>
<td>ExS127</td>
<td>5'-T CTG GAT GGA CAG AGG GGC GTG GAC ATC A-3'</td>
</tr>
<tr>
<td>K497H</td>
<td>ExS302</td>
<td>5'-CGT CTG GAT GGA CAG CAC GGC GTG GAC ATC ATC GG-3'</td>
</tr>
<tr>
<td>K497Q</td>
<td>ExS129</td>
<td>5'-T CTG GAT GGA CAG CAG GGC GTG GAC ATC A-3'</td>
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<td>K497N</td>
<td>ExS287</td>
<td>5'-CTG GAT GGA CAG AAAC GGC GTG GAC ATC-3'</td>
</tr>
<tr>
<td>K497E</td>
<td>ExS289</td>
<td>5'-CTG GAT GGA CAG GAC GGC GTG GAC ATC-3'</td>
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</table>
Expression and purification of the mutant enzymes

Enzyme expression was performed using *E. coli* mutant strain EX2 [glgA- and glgB-] as host, which allows the measurement of activities of mutant enzymes in the absence of bacterial glycogen synthase and branching enzyme. Expression and purification of maize SSIIa and its mutants were done as previously described (Imparl-Radosevich et al., 1998). Maize SSIIa and its mutants were purified to apparent homogeneity with a similar procedure as described previously (Gao et al., 2001; Imparl-Radosovich et al., 1998).

Starch synthase enzyme assay

The basic SS assay contained 100mM Bicine, pH 8.5, 5mM EDTA, 25mM potassium acetate, 10mM reduced glutathione, 0.5mg/mL BSA, 500 mM citrate and either 5mg/mL amylopectin or 20mg/mL glycogen as primer. In the citrate curve measurement, final concentrations of 0, 100, 200, 300, 400, and 500 M citrate were used for enzyme assay. ADP-glucose and primer (amylopectin and glycogen) were systematically adjusted for kinetic characterization. Reactions were initiated with the addition of [U-14C]-ADPGlc (225 µCi / mmol) into a total reaction volume of 0.1mL, and the methanol precipitation method was used to remove un-reacted [U-14C]-ADPGlc. The amount of enzyme was adjusted in each case so that a 4-minute or 8-minute reaction gave incorporation of [U-14C]-ADPGlc in the linear range (enzyme concentration versus rate) during the time course of the assay.
RESULTS

Lysine mutations result in changes in chain elongation specificity in the presence of 500 mM citrate

Characterization of the site-directed mutants of maize SSIIa demonstrated that some of the lysine mutants (K193R, K193E, K497H, K497Q, K497N, and K497E) had primer preference different from wild type, and responded differently to citrate (Table 1). Normally the SS activity assay is done in the presence of 500 mM citrate. So, that assay is the reference point for our observations. As shown in Table 1, wild type SSIIa had higher specific activity with amylopectin as primer than with glycogen as primer in the presence of 500 mM citrate, indicating that SSIIa wild type had higher preference for longer outer chains in amylopectin than shorter outer chains in glycogen. At lysine-497, all active mutants (K497H, K497Q, K497N, and K497) preferentially elongated the shorter outer chains prevalent in glycogen. K465R and K469R had the same catalytic specificity in chain elongation as wild type enzyme. At lysine-193, the mutants showed different catalytic specificities in chain elongation. Whereas mutants K193R and K193E preferentially elongated shorter outer chains, K193Q preferentially elongated longer outer chains. K193Q even showed a higher preference for elongating longer outer chains than wild type. These results clearly indicate that, different from wild type SSIIa, the mutants K193R, K193E, K497H, K497Q, K497N, and K497E preferentially elongate glycogen with shorter outer chains in the presence of 500 mM citrate. SSIIa wild type, K193R, K193Q, and K497Q were picked for further characterization.
Effect of citrate on activities of maize SSIIa and its mutants is concentration dependent

Citrate influenced activities of SSIIa wild type and its mutants in different ways. Response of enzyme activity to citrate concentrations varied, depending on type of primer and mutation (Fig. 1). With amylopectin as primer, \(A_{0.5}\) was approximately 150 mM and 110 mM citrate for wild type and K193Q, respectively. On the other hand, for K193R and K497Q, \(A_{0.5}\) could not be calculated because activity stimulation was not saturated at 500 mM citrate. Compared with activities in absence of citrate, the optimal concentration of citrate increased enzyme activities 4-fold, 3-fold, and 5-fold, respectively, for wild type, K193Q, and K193R (0.5M citrate), whereas 500 mM citrate increased the activity of K497Q about 68-fold (Fig.1; Fig. 2).

With glycogen as primer, \(A_{0.5}\) was 60 mM and 40 mM citrate, respectively, for wild type and K193Q. The activity stimulation of K193R and K497 activity was not saturated at a citrate concentration of 500 mM (Fig. 1). The stimulation degree of K497Q (60-fold) is much higher than wild type (2-fold), K193Q (1.5-fold), and K193R (3.6-fold) (Fig.1; Fig. 2).

It is noteworthy that further increase of citrate concentration reduced the enzyme activities of SSIIa wild type and K193Q after maximal stimulation was achieved (Fig. 1). This did not occur in the mutant K193R and K497Q because their activities were not saturated even at 500 mM citrate (Fig. 1). It should be mentioned that these activities were measured in the condition of saturating concentration of primer, either amylopectin or glycogen. There were differences in the activity reduction caused by high concentration of citrate between amylopectin as primer and glycogen as primer. For amylopectin as primer, the activity of SSIIa wild type in the presence of 500M citrate was 94% of maximal activity and 3.8-fold of initial activity (without citrate), and the activity of K193Q in the presence of
500 mM citrate was 71% of maximal activity and 2.3-fold of initial activity. On the other hand, for glycogen as primer, the activity of SSI1a wild type with 500 mM citrate was 45% of maximal activity and 80% of initial activity, and enzyme activity of SSI1a K193Q showed 22% of maximal activity and 32% of initial activity. Moreover, using amylopectin as primer, the activity reduction caused by high concentration of citrate started at 300–400 mM citrate; using glycogen, the inhibition started at 100–200 mM citrate. The results suggest that, in the presence of a high concentration of primer, a high concentration of citrate may impose inhibition on SSI1a wild type and K193Q, and also may impose greater inhibition on glycogen-primed activity than on amylopectin-primed activity.

The mutant K497Q was much more citrate-dependent than wild type, K193R, and K193Q. In the absence of citrate, K497Q was almost inactive (0.13 and 0.31 μmol/min/mg protein, respectively, for amylopectin as primer and glycogen as primer). Citrate greatly enhanced SS activity of K497Q by increasing its activity 60 to 68 fold (Fig. 2). The degree of stimulation was similar between activity with amylopectin as primer and activity with glycogen as primer. This implies that a high concentration of citrate may protect SSI1a enzyme from inactivation by certain mutations.

**SSIIa wild type and mutants differ in the citrate-induced switch of chain elongation specificity**

In the absence of citrate, wild type SSI1a, K193R, K193Q, and K497Q had higher catalytic preference for elongating glycogen with shorter outer chains than amylopectin with longer outer chains, indicated by catalytic chain elongation specificity ratio (A/G). Here, catalytic chain elongation specificity is measured by ratio between activity with amylopectin as primer and activity with glycogen as primer. If the ratio is less than 1, then the enzyme
has a preference for elongating shorter outer chains; if the ratio is more than 1, then the enzyme has a preference for elongating longer chains. Citrate increased the catalytic chain elongation specificity ratio (A/G) of wild type SSIIa (Fig. 3). The catalytic chain elongation specificity ratio of wild type SSIIa reached 1 at about 320 mM citrate, and reached 1.8 at 500 mM citrate, suggesting that citrate may induce the switch of catalytic chain elongation specificity of wild type SSIIa. That is, wild type SSIIa preferentially elongated shorter outer chains in glycogen when citrate concentration was less than 320 mM; however, when citrate concentration was higher than 320 mM, wild type SSIIa preferentially elongated longer outer chains in amylopectin. K193Q was more sensitive to citrate than wild type SSIIa in switching catalytic chain elongation specificity. The catalytic chain elongation specificity ratio of K193Q reached 1 at about 150 mM citrate, and reached 3.6 at 500 mM citrate (Fig. 3). The switch of catalytic chain elongation specificity occurred at lower citrate concentration for K193Q as compared with wild type, and K193Q had even higher preference for elongating longer outer chains than wild type in the presence of 500 mM citrate. However, the mutants K193R and K497Q did not change their catalytic chain elongation specificity ratios significantly with citrate concentration changing from 0 to 500 mM (Fig. 3). The two mutants showed a higher catalytic preference for shorter outer chain (glycogen) than longer outer chains (amylopectin) with and without citrate. It appears likely that substitution of glutamine for lysine-193 enhanced the property of switching catalytic chain elongation specificity with increase of citrate. On the other hand, K193R and K497Q lost the property of switching catalytic chain elongation specificity with increase of citrate.
Citrate is involved in the interaction between enzyme and primer

To determine the role of citrate in catalysis of SSIIa, ADP-glucose and glycogen kinetics of wild type and K193Q were characterized with different concentrations of citrate. ADP-glucose binding of wild type and K193Q, measured by Km for ADP-glucose, was not significantly affected by different citrate concentrations (Table 2). This suggests that citrate may not be important for ADP-glucose binding of SSIIa. Moreover, behavior of wild type at saturating (3 mM) and low concentration (0.4 mM) of ADP-glucose with or without citrate showed that different ADP-glucose concentrations did not influence catalytic primer preference (Fig. 4). This suggests that ADP-glucose does not influence catalytic primer preference of SSIIa. On the other hand, the Km for glycogen of wild type and K193Q at 150 mM citrate were eleven times and twenty eight times higher than those at 500 mM citrate, suggesting that a high concentration of citrate enhanced enzyme affinity for glycogen (Table 2). However, the activities of wild type and K193Q in the presence of 500 mM citrate were lower than those in the presence of 150 mM citrate. These results suggest that a higher concentration of citrate may enhance glycogen binding to wild type and K193Q, however simultaneously it may reduce maximal catalytic capacity of the enzymes.

It is noteworthy that the catalytic chain elongation specificity switch of wild type and K193Q was closely related to the activity reduction caused by a high concentration of citrate. This raises the question whether the activity reduction is also related to primer. In the case of amylopectin as primer, and in the presence of 500 mM citrate, wild type did not show activity reduction (Fig. 5a), whereas K193Q showed an activity drop after reaching maximal activity (about 14% activity decrease) (Fig. 5d). This result corresponds to the observation that K193Q was inhibited more, relatively, by 500 mM citrate than wild type (24% and 6%
activity decrease, respectively, for K193Q and wild type) (Fig. 1). In the case of glycogen as primer, and in the presence of 500 mM citrate, with glycogen concentration increasing the activities of wild type and K193Q showed a sharp drop after reaching the maximal activity (Fig. 5b, 5f); however, this activity reduction did not occur in K193R and K497Q (Fig. 5h, 5i). Interestingly, in the presence of 150 mM citrate (close to optimal citrate concentration for activity stimulation with glycogen as primer), the activity reduction did not occur in wild type and K193Q (Fig. 5c, 5g). This suggests that the combination of high concentration of glycogen and high concentration of citrate may reduce enzyme activity of wild type and K193Q. However, K193R and K497Q were insensitive to this type of activity inhibition. This may explain why, in the presence of 500 mM citrate K193R increased activity by 24% with glycogen as primer (Table 1).

Moreover, in the case of a low concentration of glycogen (less than 1 mg/ml glycogen), wild type and K193Q had higher activities with 500 mM citrate than with 150 mM citrate, indicating that the activities of wild type and K193Q were enhanced more by a higher concentration of citrate in the presence of a low concentration of primer. However, the citrate response was influenced by a high concentration of glycogen. The activities of wild type and K193Q with 500 mM citrate were less than those with 150 mM citrate (Fig. 6a, 6b). This observation implies that citrate may function in different ways under different conditions. Mutant K193Q was observed to be more sensitive to the interaction of citrate and primer as compared with wild type. With a very low concentration of glycogen (0.05 and 0.1 mg/ml), K193Q had a higher activity ratio between 500 mM citrate and 200 mM citrate than wild type; on the other hand, with a high concentration of glycogen (≥ 1 mg/ml), K193Q had a lower activity ratio between 500 mM citrate and 200 mM citrate than wild type (Fig. 6c).
DISCUSSION

There are two soluble starch synthase peaks in anion-exchange chromatography; namely, SS activity peak I and peak II (Boyer and Preiss, 1981). A significant difference between SS activity peak I and peak II is that, in the absence of citrate, peak I has higher preference for glycogen as primer than amylopectin, whereas peak II has higher preference for amylopectin as primer than glycogen (Sivak and Preiss, 1998). So far, four maize soluble starch synthase isoforms have been identified and characterized (Harn et al., 1998; Knight et al., 1998; Gao et al., 1998; Imparl-Radosevich et al., 1998; 1999). Characterization of the recombinant SSI has shown that SSI has higher preference for glycogen than amylopectin regardless of the presence of citrate (Imparl-Radosevich et al., 1998). Clearly, SSI corresponds to SS activity peak I. For SSIIa, our study has demonstrated that SSIIa prefers elongating shorter outer chains in the absence of citrate whereas this is reversed in the presence of 500 mM citrate. That is, in terms of catalytic chain elongation specificity, SSIIa appears a SS activity peak I-like starch synthase in the absence of citrate, and high concentration of citrate can make SSIIa be an activity peak II-like starch synthase by switching its primer preference. The recombinant SSIIb may be quite different. SSIIb prefers elongating shorter outer chains in presence of 500 mM citrate, which is similar to catalytic primer preference of activity peak I (Imparl-Radosevich et al., 1999). However, it is not known whether SSIIb exhibits the same catalytic chain elongation specificity in the absence of citrate as that in the presence of 500 mM citrate. It is unknown whether Du1 has the same catalytic chain elongation specificity as SSII activity peak although it has been proposed that, based on protein molecular mass and transcription pattern, activity peak II is coded by Du1. Interestingly, SSI does not switch its catalytic chain elongation specificity
with change in citrate concentration, whereas SSIIa shows the property of switch in catalytic chain elongation specificity with change in citrate concentration.

It has been known that citrate can stimulate starch synthase activity and decrease $K_m$ for primer *in vitro* (Ozbun et al., 1971; Imparl-Radosevich et al., 1998, 1999). Furthermore, our *in vitro* study has demonstrated that citrate may also regulate catalytic chain elongation specificity for starch synthase. In the absence of citrate, both SSI (Imparl-Radosevich et al., 1998) and SSIIa preferentially elongate the shorter outer chains; but based on the catalytic primer preference ratio (A/G), SSI may have a higher catalytic preference for elongating shorter outer chains than SSIIa (Fig. 7). A high concentration of citrate may switch catalytic chain elongation specificity of SSIIa from shorter outer chains to longer outer chains. Similarly, a high concentration of citrate increases catalytic chain elongation specificity ratio (A/G) of SSI twofold over no citrate (Fig. 7). That is, citrate may also enhance the capacity of SSI wild type for elongating longer outer chains in amylopectin although 500 mM citrate does not switch the chain elongation specificity of SSI (Fig. 7). Collectively, *in vitro*, citrate can enhance the catalytic capacity of SSI and SSIIa for elongating longer outer chains, and SSIIa is much more sensitive than SSI to the regulation of catalytic specificity in chain elongation by citrate. However, we are cautious about extending this *in vitro* result to speculations of *in vivo* regulation of starch synthases because of the difference in citrate concentration between *in vivo* and *in vitro* conditions. Although *in vivo* citrate concentration is not precisely determined, it is believed that *in vivo* citrate concentration in maize endosperm is less than the *in vitro* citrate concentration (500 mM) usually used in SS enzyme assay (Liu and Shannon, 1981). It is very likely that the influence of *in vivo* citrate on starch
synthases may be similar with that of a low concentration of citrate demonstrated in this research.

Citrate is involved in the interaction between primer and enzyme rather than the interaction between ADP-glucose and enzyme. Our study has shown that citrate did not affect the ADP-glucose binding of SSIIa wild type, and in addition different ADP-glucose concentrations did not alter primer preference of wild type (Table 2; Fig. 5). Hence, the possibility of the interaction of citrate and ADP-glucose can be excluded. On the other hand, citrate may influence the chain elongation specificity of SSIIa through affecting the interaction between primer and the enzyme. A high concentration of citrate significantly decreased Km for primer glycogen, suggesting that high concentrations of citrate can enhance glycogen binding of SS. Citrate could enhance primer binding to the enzyme through stabilizing enzyme-primer complex with its carboxyl group (Nichols, 2000). However, citrate may influence primer affinity and catalytic capacity in different ways. Wild type SSIIa achieves maximal catalytic capacity at about 150 mM citrate, but its affinity to glycogen is far from optimum; a higher concentration of citrate (500 mM) reduces catalytic capacity whereas it simultaneously enhances primer binding. Moreover, it is noteworthy that high concentrations of citrate stimulated SS activities more sharply than low concentrations in the case of low glycogen concentration; on the other hand, a low concentration of citrate allows SS to achieve greater stimulation of citrate in the case of a high glycogen concentration (Fig. 6). The combination of a high concentration of citrate and a high concentration of primer may result in activity inhibition after reaching the maximal activity, which particularly occurred in wild type and K193Q with glycogen as primer (Fig. 5). A possible explanation is that citrate enhances binding of primer to the enzymes and therefore
promotes the reaction of elongation when supply of primer is a limiting factor; however, when primer concentration is high, stronger binding of primer to enzyme induced by citrate might affect conformation of the active site of SSIIa, and consequently influence its catalytic capacity as well as catalytic chain elongation specificity. Although the mechanism by which citrate is involved in the interaction between enzyme and primer is not clear, it seems that the interaction between citrate and glycogen may be either cooperative or inhibitive, largely depending on the combination of their relative concentrations.

The mutations at the conserved lysine residues of SSIIa may change its property of chain elongation specificity switching induced by citrate. Mutants K193R and K497Q lost this property of citrate-induced switch of chain elongation specificity. However, K193Q seems more sensitive to citrate regulation (Fig. 3). Further characterization of wild type, K193R, K193Q, and K497Q has demonstrated two different patterns of response to citrate (Table 3). It is noteworthy that SS activity of K497Q became much more dependent on citrate stimulation than that of wild type and another mutants (Fig. 2), and activity inhibition by high concentrations of citrate in the presence of saturated glycogen did not occur in K497Q. This suggests that the substitution of glutamine for lysine-497 may change the interaction between enzyme and primer. Two mutations at lysine-193 led to two extremes. The substitution of arginine for lysine-193 caused insensitivity of SSIIa to regulation of chain elongation specificity by citrate; the substitution of glutamine for lysine-193 made SSIIa be more sensitive to citrate regulation. It is very likely that lysine-193 and lysine-497 may be involved in the interaction between SSIIa and citrate.

In summary, wild type SSIIa preferentially elongates shorter outer chains in the absence of citrate; citrate may induce the switch of SSIIa catalytic specificity in chain
elongation from shorter outer chains to longer outer chains. This is a significant difference in catalytic property between SSIIa and SSI. SSI preferentially elongates shorter outer chains regardless of presence of citrate. The SSIIa mutants K193R and K497Q lose the property of citrate-induced switch of catalytic chain elongation specificity, and become SSI-like enzymes in terms of response to citrate in catalytic chain elongation specificity.

Soluble starch synthases show different catalytic preference for different outer chain length, which may influence elongation of outer chains of different length and then affect starch structure. SSI and SSIIa can elongate shorter chains of glycogen (average OCL= 6.5) and longer chains of amylopectin (average OCL = 11.5) with different preferences. But, SSIIa has a higher catalytic preference for the longer outer chains prevalent in amylopectin than does SSI. It is likely that SSIIa contributes more to elongation of intermediate chains, as compared with SSI. Although the in vivo regulation of SSIIa by citrate is not known yet, our in vitro research shows that citrate could regulate starch biosynthesis by affecting primer binding to SSIIa, enzyme catalytic capacity, and chain elongation specificity.

REFERENCES

203, 237-244.


Table 1. Activities of SSIIa and its mutants with 0.5 M citrate and without citrate (mean ± standard error)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>500 mM Citrate</th>
<th>No citrate</th>
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<tbody>
<tr>
<td></td>
<td>Amylopectin as primer</td>
<td>Glycogen as primer</td>
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<tr>
<td>SSIIa wild type</td>
<td>47.63±2.03</td>
<td>37.36±2.40</td>
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<tr>
<td>K193R</td>
<td>18.29±0.47</td>
<td>41.29±0.91</td>
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<td>21.01±0.04</td>
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<td>K193E</td>
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<tr>
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<td>K497E</td>
<td>4.38±0.25</td>
<td>5.69±0.26</td>
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NA—assays have not been done.
Table 2. Enzyme Kinetics of maize SSIIa wild type and K193Q with high and low concentration of citrate (Mean ± SE)

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<th>Citrate concentration</th>
<th>Vmax</th>
<th>SSIIa wild type Km for ADPG</th>
<th>Km for glycogen</th>
<th>Vmax</th>
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<td>High citrate concentration. (500 mM)</td>
<td>37.36±1.10</td>
<td>0.127±0.003</td>
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<td>Low citrate concentration. (150 mM)</td>
<td>54.35±1.66</td>
<td>0.117±0.007</td>
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Table 3. Different Citrate Response Patterns of SSIIa and Its Mutants

<table>
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<th>Type I</th>
<th>Type II</th>
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<td></td>
<td>SSIIa wt, K193Q</td>
<td>K193R, K497Q</td>
</tr>
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<td>Primer preference without citrate</td>
<td>Glycogen</td>
<td>Glycogen</td>
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<tr>
<td>Primer preference at 0.5M citrate</td>
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<td>Glycogen</td>
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<td>Enzyme activity inhibition by high conc. of glycogen in presence of 0.5M citrate</td>
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<td>No</td>
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<tr>
<td>Optimal citrate concentration maximal activity stimulation</td>
<td>Amylopectin: 300–400 mM Glycogen: 100–200 mM</td>
<td>Amylopectin: not saturated at 500 mM Glycogen: not saturated at 500 mM</td>
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<tr>
<td>Maximal citrate stimulation</td>
<td>Amylopectin: 3–4 fold Glycogen: 1.5–2 fold</td>
<td>Amylopectin: K193R 5 fold K497Q 65 fold Glycogen: K193R 4 fold K497Q 60 fold (at 500 mM citrate)</td>
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<tr>
<td>Activity reduction by high concentration of citrate (compared with max activity)</td>
<td>Amylopectin: inhibition starts at 300–400 mM citrate. Activity decreased 6–29% at 0.5M citrate. Glycogen: inhibition starts at 200–300 mM citrate. Activity decreased 55–78% at 500 mM citrate.</td>
<td>Not observed ≤ 500 mM citrate.</td>
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</table>
Figure 1. Change in specific activities of maize SSIIa wild type, K193Q, K193R, and K497Q with change in citrate concentration in the assay medium and with either amylopectin (●) or glycogen (■) as primer. (a) SSIIa wild type, (b) K193Q, (c) K497Q, and (d) K193R.
Figure 2. Change in relative activities of maize SSIIa wild type, K193Q, K193R, and K497Q with change in citrate. The relative activity is defined as follows. The activity (0.0 mM citrate) of an enzyme with a certain primer is set to be 100%, and the activities of the enzyme with the primer at different citrate concentrations is a percentage of the initial.
Figure 3. Change in primer preference of maize SSIIa and its mutants with citrate concentration. Primer preference ratio is defined as follows: Primer preference ratio = activity with amylopectin ÷ activity with glycogen.
Figure 4. Influence of citrate on the primer preference of SSIIa wild type and K193Q at low and high concentration of ADP-glucose
Figure 4. Activity curves of SSIa wild type, K193Q, K193R, and K497Q with different concentrations of primer. (a) SS activity curve of wild type with different concentration of amylopectin at 500 mM citrate; (b) SS activity curve of wild type with different concentration of glycogen at 500 mM citrate; (c) SS activity of wild type with different concentration of glycogen at 150 mM citrate; (d) SS activity of K193Q with different concentration of amylopectin at 500 mM citrate; (e) SS activity of K193Q with different concentration of glycogen at 500 mM citrate; (f) SS activity of K193Q with different concentration of glycogen at 150 mM citrate; (g) SS activity of K497Q with different concentration of glycogen at 500 mM citrate; (i) SS activity of K193R with different concentration of glycogen at 500 mM citrate.
Figure 6. Influence of high and low concentrations of citrate on the specific activities of SSIIa wild type and K193Q at different concentration of glycogen; Ratio of activities with 500 mM citrate to that with 200 mM citrate.
Fig. 7. Primer preference ratio (activity with amylopectin as primer + activity with glycogen as primer) of SSI and SSIIa without citrate and with 500 mM citrate (Raw data for SSI is from Imparl-Radosевич et al., 1998)
Chapter 5. General Discussion and Conclusions

Starch plays a very important role in human nutrition and various kinds of industrial application. An improved understanding of the mechanism of starch biosynthesis will greatly facilitate traditional breeding and genetic engineering to improve starch quantity and quality.

Whereas the structure-function relationship of ADP-glucose pyrophosphorylase and branching enzymes has been intensively studied, not much is known about the structure-function relationship of starch synthases. The most significant hypothesis on the structure-function relationship of plant starch synthases is derived from the research on the enzyme mechanisms of \textit{E.coli} glycogen synthase. Furukawa et al. (1990; 1993) identified lysine-15 of the conserved K-T-G-G motif as the substrate ADP-glucose binding site of \textit{E.coli} glycogen synthase using affinity labeling and site-directed mutagenesis. Plant starch synthase and \textit{E.coli} glycogen synthase catalyze the same reaction of \(\alpha\)-1, 4-glucan elongations. Both enzymes use ADP-Glucose as a substrate and transfer glucose to the non-reducing end of \(\alpha\)-glucan. Furthermore, the K-X-G-G motif is highly conserved between \textit{E.coli} glycogen synthase and plant starch synthases (including all isoforms of maize starch synthase); hence it has become widely accepted that the lysine of the conserved K-X-G-G motif may also function as the ADP-glucose binding site in plant starch synthases. My results are at variance with this assumption. In this study, three mutants, K193R, K193Q and K193E, of SSIIa were made at lysine-193 to determine its function in maize. My results were not consistent with the popular hypothesis that lysine-193 of the conserved K-T-G-G motif is involved in ADP-glucose binding of maize SSIIa. The mutations at the conserved lysine-193 (corresponding to lysine15 of \textit{E.coli} glycogen synthase) did not change the ADP-
glucose affinity of maize SSIIa. In contrast with *E. coli* glycogen synthase, the ε-amino group of lysine-193 is not involved in the binding of ADP-glucose in maize SSIIa because replacement of glutamine or glutamic acid for lysine-193 did not affect the Km for ADP-glucose of maize SSIIa. On the other hand, the mutations at lysine-193 did influence enzyme activity of maize SSIIa, indicating that lysine-193 is involved in catalysis of maize SSIIa rather than ADP-glucose binding.

I examined whether the functional difference in the lysine residue of K-T-G-G between *E. coli* and maize SSIIa is caused by a structural difference between the two enzymes. A major structural difference between *E. coli* glycogen synthase and maize SSIIa is that maize SSIIa possesses an N-terminal extension of 176 amino acids. My kinetic characterization has shown that the N-terminal extension of maize starch synthase Ila does not influence the function of the lysine-193 of K-T-G-G in terms of ADP-glucose affinity.

Chemical modification results have demonstrated that lysine is involved in ADP-glucose binding of SSIIa. This raised the question: which lysine residue(s) is responsible for ADP-glucose binding of maize SSIIa. Apart from lysine-193, three other highly conserved lysine residues have been identified in maize SSIIa; i.e., lysine-465, lysine-469, and lysine-497. Kinetic characterization of the purified SSII mutants K465R and K469R showed that substitution of arginine for lysine-465 or lysine-469 did not change ADP-glucose affinity. However, the possibility of involvement of lysine-465 and lysine-469 in ADP-glucose binding cannot be ruled out because substitution of glutamine or glutamic acid resulted in complete loss of enzyme activity.

Site-directed mutagenesis and kinetic characterization have demonstrated that lysine-497 is directly involved in ADP-glucose binding of maize SSIIa. Although the replacement
of lysine with arginine (K497R) or histidine (K497H) resulted in much less activity than the replacement of lysine with glutamine (K497Q), or asparagine (K497N), or glutamic acid (K497E), the mutations K497Q, K497N, and K497E led to more serious deterioration in ADP-glucose binding of SSIIa than K497H. It is worth noting that the replacement of lysine-497 with histidine caused almost complete loss of activity. However, the replacement induced a much smaller Km for ADP-glucose than did other mutations (K497Q, K497N, and K497E). This suggests that the positive charge of lysine-497 is important for substrate ADP-glucose binding, and size restriction operates on catalysis at amino acid position 497. Moreover, the effects of K497Q are not caused by a global conformational change, as defined by CD spectra. Hence, I believe that the conserved lysine-497 is a likely candidate for involvement in ADP-glucose binding of maize SSIIa.

A three-dimensional structure model of SSIIa is proposed based on protein threading to better understand the structure-function relationship of starch synthases. In our protein threading, maize starch synthase IIa fits to UDP-N-acetylglucosamine 2-epimerase from *E.coli* with the highest confidence. In this model, lysine-193, lysine-497, and aspartic acid 199 (Nichols et al., 2000) of maize SSIIa geometrically correspond to lysine-15, histidine-213, and glutamic acid 296 of UDP-N-acetylglucosamine 2-epimerase, respectively. Lysine-15, histidine-213, and glutamic acid 296 are located in the active site of UDP-N-acetylglucosamine 2-epimerase with important function. Glu-296 has been confidently assigned a role in binding the UDP portion of the substrate. Lysine-15 and histidine-213 are found in the active site. The role of these residues well matches the observed function of conserved lysine-193, aspartic acid 199, and lysine-497 in maize SSIIa. The likely scenario
of catalysis and substrate binding in maize SSIIa is that lysine-193 is involved in catalysis, and lysine-497 and aspartic acid 199 participate in substrate ADP-glucose binding of SSIIa.

The dissertation also addresses an influence of citrate on catalytic chain elongation specificity of maize SSIIa. A significant difference between SS activity peak I and peak II is that, in the absence of citrate, peak I has a higher preference for glycogen as primer than amyllopectin, whereas peak II has a higher preference for amyllopectin as primer than glycogen (Boyer and Preiss, 1981; Sivak and Preiss, 1997). The recombinant SSI preferentially elongates shorter outer chains in glycogen regardless of the presence of citrate (Imparl-Radosevich et al., 1998). Hence, SSI corresponds to SS activity peak I. My study has demonstrated that SSIIa preferentially elongates shorter outer chains in the absence of citrate, whereas the enzyme preferentially elongates longer outer chains in the presence of 0.5 M citrate. That is, in terms of chain elongation specificity, SSIIa behaves like a SS activity peak I starch synthase in the absence of citrate, whereas it behaves like a SS activity peak II starch synthase in the presence of 0.5 M citrate.

Our in vitro study has demonstrated that citrate may also regulate catalytic chain elongation specificity of starch synthase. In the absence of citrate, both SSI (Imparl-Radosevich et al., 1998) and SSIIa preferentially elongate the shorter outer chains of glycogen two to three times faster than the longer outer chains of amyllopectin, but the catalytic chain elongation specificity ratio (A/G) of SSIIa is higher than that of SSI. This implies that SSI has a higher catalytic preference for elongating the shorter outer chains prevalent in glycogen than does SSIIa in the absence of citrate. A high concentration of citrate may switch catalytic chain elongation specificity of SSIIa from shorter outer chains to longer outer chains. Similarly, a high concentration of citrate increases chain elongation
specificity ratio (A/G) of SSI twofold over no citrate. Collectively, \textit{in vitro}, citrate can enhance the catalytic capacity of SSI and SSIIa for elongating longer outer chains, and SSIIa is much more sensitive than SSI to the regulation of catalytic specificity in outer chain elongation by citrate.

The mutations at the conserved lysine residues of SSIIa may change its property of citrate-induced switch of chain elongation specificity. Mutants K193R and K497Q lose the property of citrate-induced chain elongation specificity switch. However, K193Q seems more sensitive to citrate regulation. SS activity of K497Q became much more dependent on citrate stimulation than that of wild type and another mutants, suggesting that the substitution of glutamine for lysine-497 may change the interaction between enzyme and primer. Two mutations at lysine-193 led to two extremes. The substitution of arginine for lysine-193 caused SSIIa to be insensitive to regulation of chain elongation specificity by citrate; the substitution of glutamine for lysine-193 made SSIIa more sensitive to citrate regulation. It is very likely that lysine-193 and lysine-497 may be involved in the interaction between SSIIa and citrate.

Citrate is involved in the interaction between primer and enzyme rather than the interaction between ADP-glucose and enzyme. My study has shown that citrate did not affect the ADP-glucose binding of SSIIa wild type, and in addition different ADP-glucose concentrations did not alter primer preference of wild type. Hence, the possibility of the interaction of citrate and ADP-glucose can be excluded. On the other hand, citrate may influence the chain elongation specificity of SSIIa by affecting the interaction between the enzyme and primer. A high concentration of citrate significantly decreased Km for primer glycogen, suggesting that high concentrations of citrate can enhance glycogen binding of SS.
Citrate could enhance primer binding to the enzyme through stabilizing the enzyme-primer complex with its carboxyl group (Nichols, 2000). It is noteworthy that wild type SSIIa achieves maximal catalytic capacity at about 0.15 M citrate, but its affinity for glycogen is far from optimum; a higher concentration of citrate (0.5 M) reduces catalytic capacity whereas it simultaneously enhances primer binding. A possible explanation is that citrate enhances binding of primer to enzymes and therefore promotes the reaction of elongation when supply of primer is a limiting factor; however, when primer concentration is high, stronger binding of primer to enzyme induced by citrate might affect conformation of the active site of SSIIa, and consequently influence its catalytic capacity as well as chain elongation specificity. Although the mechanism by which citrate is involved in the interaction between enzyme and primer is not clear, it seems that the interaction between citrate and primer may be either cooperative or inhibitive, largely depending on the combination of their relative concentrations.

References


