Role of the N-Terminal Starch-Binding Domains in the Kinetic Properties of Starch Synthase III from *Arabidopsis thaliana*[†]

Hugo A. Valdez,[‡] Maria V. Busi,[‡] Nahuel Z. Wayllace,[‡] Gustavo Parisi,[§] Rodolfo A. Ugalde,[‡] and Diego F. Gomez-Casati*[,‡]

Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), CONICET/UNSAM, Camino Circunvalación Km 6, 7130 Chascomús, Argentina, and Centro de Estudios e Investigaciones, Universidad Nacional de Quilmes, Roque Saenz Peña 352, B1876BXD Bernal, Buenos Aires, Argentina

Received December 11, 2007

ABSTRACT: Starch synthase III (SSIII), one of the SS isoforms involved in plant starch synthesis, has been reported to play a regulatory role in the synthesis of transient starch. SSIII from *Arabidopsis thaliana* contains 1025 amino acid residues and has an N-terminal transit peptide for chloroplast localization which is followed by three repeated starch-binding domains (SBDs; SSIII residues 22−591) and a C-terminal catalytic domain (residues 592−1025) similar to bacterial glycogen synthase. In this work, we constructed recombinant full-length and truncated isoforms of SSIII, lacking one, two, or three SBDs, and recombinant proteins, containing three, two, or one SBD, to investigate the role of these domains in enzyme activity. Results revealed that SSIII uses preferentially ADPGlc, although UDPGlc can also be used as a sugar donor substrate. When ADPGlc was used, the presence of the SBDs confers particular properties to each isoform, increasing the apparent affinity and the V_{max} for the oligosaccharide acceptor substrate. However, no substantial changes in the kinetic parameters for glycogen were observed when UDPGlc was the donor substrate. Under glycogen saturating conditions, the presence of SBDs increases progressively the apparent affinity and V_{max} for ADPGlc but not for UDPGlc. Adsorption assays showed that the N-terminal region of SSIII, containing three, two, or one SBD module have increased capacity to bind starch depending on the number of SBD modules, with the D23 protein (containing the second and third SBD module) being the one that makes the greatest contribution to binding. The results presented here suggest that the N-terminal SBDs have a regulatory role, showing a starch binding capacity and modulating the catalytic properties of SSIII.

Starch, the carbohydrate storage form in plants, serves as the main source of energy storage in these organisms. It is formed by two major components: amylose, which is predominantly a linear α-1,4-glucan chain, and amylopectin, a highly branched α-1,4-α-1,6-glucan (*1, 2*).

The metabolic pathways leading to the biosynthesis of these polymers involve the action of several enzymes, such as ADP-glucose pyrophosphorylase (ADPGlc PPase,[1] EC 2.7.7.27), starch synthase (SS, EC 2.4.1.21), and branching enzyme (BE, EC 2.4.1.18). Both polymers are synthesized from ADPGlc, the sugar nucleotide donor. SS catalyzes the extension of the α-1,4 chain of starch, transferring a glycosyl unit from the ADPGlc to the nonreducing end of a preexisting α-1,4 polymer (*1–3*).

Multiple isoforms of SS have been described in plants: the soluble forms named SSI, SSII, SSIII, SSIV, and SSV and the granule-bound SS, GBSSI and GBSSII. However, only five loci corresponding to starch synthases have been detected in the genome of *Arabidopsis thaliana*: AtGBS1 (At1g32900), AtSS1 (At5g24300), AtSS2 (At3g01180), AtSS3 (At1g11720), and AtSS4 (At4g18240). These loci encode GBSSI, SSI, SSII, SSIII, and SSIV isoforms, respectively (*4*). The different SS isoenzymes, together with BE, play an important role in the final structure of starch. Furthermore, it has been suggested that each SS isoform has a specific role in the starch biosynthetic pathway (*3, 5, 6*). The granule-bound isoenzymes are mainly responsible for the synthesis of the amylose fraction. SSI has been suggested to be mainly involved in the synthesis of small chains of amylopectin. SSII and SSIII play a major role in the synthesis of this fraction, whereas it has been suggested that SSIV has a specific function in the control of the number of granules (*4, 6–8*).

The role of the SSIII isoform in the synthesis of starch has been less studied. It has been reported that *Arabidopsis* null mutants lacking SSIII activity have a starch excess phenotype

[†] This work was partially supported by grants from ANCYPT 01-13926 and the Biotechnology Program of UNSAM. H.A.V. is a doctoral fellow from CONICET. N.Z.W. is a doctoral fellow from ANPCyT. G.P., M.V.B., R.A.U., and D.F.G.-C. are research members from CONICET.

* To whom correspondence should be addressed. E-mail: diego.gomezcasati@intech.gov.ar. Telephone: +54 2241430323. Fax: + 54 2241 424048.

[‡] CONICET/UNSAM.

[§] Universidad Nacional de Quilmes.

[1] Abbreviations: SSIII, starch synthase III; ADPGlc, adenosine diphosphoglucose; UDPGlc, uridine diphosphoglucose; ADP-glucose PPase, ADPGlc pyrophosphorylase; BE, branching enzyme; SBD, starch-binding domain; SS, starch synthase; GBSS, granule-bound starch synthase; CBM, carbohydrate-binding module; IPTG, isopropyl β-D-thiogalactopyranoside; GS, glycogen synthase; SSIII-CD, starch synthase III catalytic domain; LB, Luria-Bertani; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; K_{ad}, adsorption constant; MOPS, 3-(*N*-morpholino) propanesulfonic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium.

10.1021/bi702418h CCC: $40.75
Published on Web 02/09/2008

due to an apparent increase in the rate of starch synthesis, suggesting that SSIII has a negative regulatory role in the biosynthesis of this polymer (*6*). Recently, the characterization of a SSIII from kidney bean provided evidence for the function of the N-terminal region as a carbohydrate-binding module (*9*). However, the relation between structure and function of this SS isoform is far from being complete.

Members of the starch synthase family display a high degree of sequence similarity in their C-terminal catalytic domain. However, among the isoforms, the differences are mainly present at the N-terminal domain (*10*). Bioinformatics analyses revealed that the SSIII from *A. thaliana* encodes three putative starch-binding domains (SBDs) at its N-terminal region (*11*). SBDs have been described as noncatalytic modules, related to the carbohydrate-binding module (CBM) family. In general, all carbohydrate-binding modules (CBMs) have been classified into the CBM families. In particular, on the basis of sequence similarities, individual types of SBDs have been placed into seven CBM families: CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, and CBM45 (*12*). All the information is available at the CAZy web server (http://www.cazy.org/CAZY/).

On the basis of bioinformatic analyses, SBDs from SSIII have been grouped in the CBM21 family (*13*). The presence of SBDs has been described in several bacterial and fungal enzymes participating in starch degradation such as amylases, glucoamylases, and pullulanases (*14, 15*) as well as in other mammalian enzymes (*16*). Although there are only few reports about the characterization of SSIII or SBDs in higher plants, the possible regulatory role of SSIII makes this isoform a potential protein target for manipulating the level and quality of starch in plants.

SSIII from *A. thaliana* (At1g11720, GenBank accession number EF636491) is a 1025-amino acid residue protein containing different regions: (i) a putative transit peptide of 21 amino acids, (ii) an N-terminal SSIII isoform specific region [specific domain (SSIII-SD), residues 22−591], and (iii) a C-terminal catalytic domain, which is common to all isoforms of starch synthases (residues 592−1025). This arrangement has also been described in other plants (*9, 10, 17, 18*). As described above, SSIII-SD contains three internal repeats, named D1, D2, and D3, that encode putative SBDs (Figure 1). We have previously compared the SSIII D2 domain to the SBD of the glucoamylase from *Aspergillus niger* and proposed a three-dimensional model (*11*). However, the biochemical function of these SBDs is as yet unknown.

In this work, we investigated the function of the SBDs present on the N-terminal region of *A. thaliana* SSIII. For this purpose, the full-length enzyme as well as truncated isoforms lacking one, two, or three SBDs (and also the SBD proteins) was cloned, expressed, and purified from *Escherichia coli* cells, and their kinetic parameters were studied. On the basis of our results, we propose that the N-terminal SBDs have a regulatory role in SSIII activity, showing starch binding ability and modulating the catalytic properties of SSIII.

RESULTS

Cloning, Expression, and Purification of A. thaliana SSIII Wild Type and Truncated Isoforms and Starch-Binding Domain Proteins. To explore the function of SBDs, different SSIII constructs containing three N-terminal SBDs (SSIII-D123), two SBDs (SSIII-D23), or one SBD (SSIII-D3) or lacking any SBD (SSIII catalytic domain, SSIII-CD) (*19*) were cloned and expressed in *E. coli.* Furthermore, the individual SBD proteins (D123, D23, D2, and D3) were also cloned and expressed (Figure 1). Each truncated isoform of SSIII and SBD proteins were generated by PCR using internal primers as described in Experimental Procedures (see also Figure 1). Inserts were cloned in the pET-32c expression vector (Novagen) containing a C-terminal His_6 tag. Constructs pVAL15 (containing SSIII-D123), pVAL17 (containing SSIII-D23), pVAL18 (containing SSIII-D3), pVAL3 (containing SSIII-CD), pVAL19 (containing D123), pVAL20 (containing D23), pVAL21 (containing D3), and pVAL25 (containing D2) (Figure 1) were submitted to restriction mapping and DNA sequencing to control PCR fidelity. The full-length *Arabidopsis* SSIII isoform, SSIII-D123, contains 1012 amino acid residues but lacks the N-terminal transit peptide for chloroplast localization. SSIII-D23, SSIII-D3, and SSIII-CD contain 744, 578, and 458 amino acid residues, respectively. Proteins containing the different SBD regions, D123, D23, D2, and D3, contain 554, 286, 166, and 120 amino acid residues, respectively.

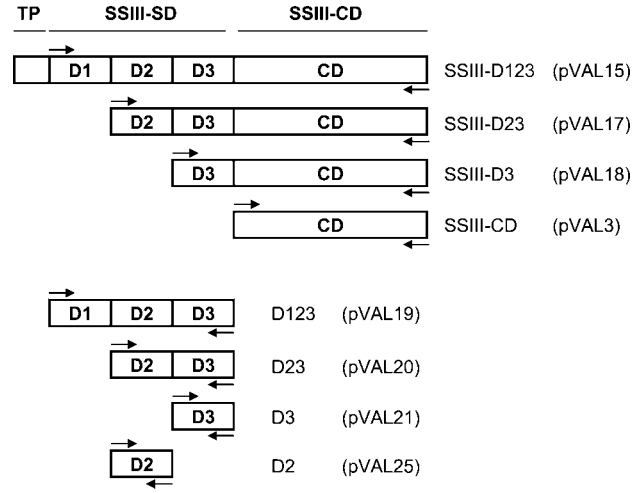

FIGURE 1: Structure domain of SSIII and SBD proteins from *A. thaliana*. Arrows show the locations and directions of the primers used to clone the different enzymes: TP, transit peptide; SSIII-SD, starch synthase III specific domain; SIII-CD, starch synthase III catalytic domain; D1−D3, three tandem modules containing the starch-binding domains; SSIII-D123, full-length enzyme, lacking the transit peptide and containing the three SBDs; SSIII-D23, truncated isoform lacking the D1 domain; SSIII-D3, protein lacking D1 and D2; SSIII-CD, C-terminal catalytic domain, lacking all the SBDs. The names of each final construct are on the right side of the figure (in parentheses).

All the constructs were expressed in BL21(DE3)-RIL cells and induced with IPTG. Proteins were purified to apparent homogeneity by a single purification step using a HiTrap chelating column (Amersham Biosciences) equilibrated with 0.1 M $NiSO_4$. This procedure yielded 0.1 mg of purified SSIII-D123 and SSIII-D23 per gram of cells, 0.6 mg of purified SSIII-D3 and D2, and 0.2 mg of purified SSIII-CD, D123, D23, and D3 per gram of cells, respectively.

Panels A, C, and E of Figure 2 show the SDS−PAGE analysis of recombinant SSIII and SBD proteins eluted from the affinity chromatography column. A single protein band for each isoform, with the expected molecular mass, was eluted from the column in the range between 150 and 250 mM imidazole. Thus, we consider that all SSIII isoforms are homogeneous after this purification procedure. The

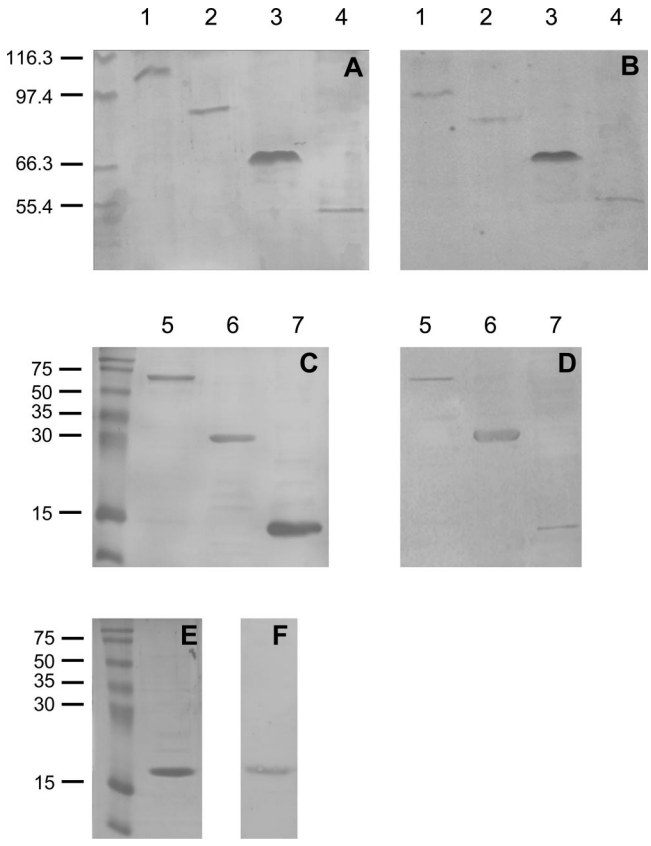

FIGURE 2: (A) SDS−PAGE of recombinant SSIII enzymes: lane 1, SSIII-D123; lane 2, SSIII-D23; lane 3, SSIII-D3; and lane 4, SSIII-CD. Numerals indicate molecular masses of the following standards: β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), BSA (66.3 kDa), and glutamic dehydrogenase (55.4 kDa). (B) Western blot detection of purified recombinant SSIII proteins shown in panel A using anti-His antibodies. The molecular mass standards used are the same as those used in panel A. (C) SDS−PAGE of recombinant SBDs proteins: lane 5, D123; lane 6, D23; and lane 7, D3. Numerals indicate molecular masses of the standards (Rainbow Molecular Weight Markers, Amersham Biosciences). (D) Western blot detection of SBD proteins shown in panel C. Panels E and F show SDS−PAGE and Western blotting of recombinant D2 protein, respectively.

absence of the endogenous glycogen synthase (GS) was investigated by Western blotting using antibodies raised against recombinant His_6-tagged GS from *E. coli* and by measuring the enzymatic activity. It was determined that the bacterial enzyme elutes from the Ni chelating column during the washing step with buffer A. Thus, this procedure completely separates the small amount of endogenous *E. coli* GS from each recombinant SS isoform (data not shown).

We confirmed the presence of the recombinant SSIII full-length and truncated proteins by immunoblotting using penta-His antibodies that react with the C-terminal His_6 tag (Figure 2B). We also analyzed the presence of SBD proteins using the same procedure (Figure 2D,F). As reported for SSIII-CD (*19*), all the SSIII isoforms cross-reacted with antibodies raised against *Agrobacterium tumefaciens* glycogen synthase, confirming the existence of the structural similarity reported between both enzymes (data not shown).

Effect of SBDs on the Kinetic Parameters of Starch Synthase III for the Polysaccharide Substrate. Table 1 shows the enzymatic activity of the wild-type full-length and truncated forms of SSIII using glycogen, amylose, or amylopectin as the acceptor oligosaccharide. The full-length enzyme exhibited a higher activity with glycogen than with amylose or amylopectin (11- or 4-fold higher, respectively). Similar results, showing the highest activity with glycogen, were obtained for SSIII-D23 and SSIII-D3 and previously with the truncated form, SSIII-CD (Table 1) (*19*). We have also reported that UDPGlc is a good substrate for SSIII-CD (*19*). Interestingly, all the SSIII isoforms exhibit activity with UDPGlc. Thus, we study the kinetic parameters of the different SSIII isoforms using glycogen as the acceptor polysaccharide and ADPGlc or UDPGlc as the glycosyl donor.

Table 1: Specific Activities of SSIII Full-Length and Truncated Proteins in the Presence of Different Polysaccharide Substrates

	specific activity (units/mg)		
isoform	glycogen	amylose	amylopectin
SSIII-D123	5.62 ± 0.53	0.51 ± 0.04	1.26 ± 0.15
SSIII-D23	5.19 ± 0.37	0.45 ± 0.13	1.20 ± 0.21
SSIII-D3	0.75 ± 0.14	0.14 ± 0.09	0.33 ± 0.10
SSIII-CD[a]	0.06 ± 0.01	0.02 ± 0.004	0.03 ± 0.01

[a] Data obtained from ref (*19*).

Table 2: Kinetic Parameters of SSIII Proteins Using Saturating Concentrations of ADPGlc or UDPGlc and Glycogen as the Acceptor Polysaccharide

isoform	$S_{0.5}$ (mg/mL)	n_H	V_{max} (units/mg)	$V_{max}/S_{0.5}$
	(A) ADPGlc			
SSIII-D123	0.26 ± 0.08	0.9 ± 0.4	5.99 ± 0.48	23.03 ± 4.31
SSIII-D23	0.68 ± 0.11	1.4 ± 0.3	4.92 ± 0.52	7.24 ± 1.93
SSIII-D3	1.92 ± 0.34	1.1 ± 0.2	0.78 ± 0.15	0.41 ± 0.15
SSIII-CD	2.65 ± 0.21	1.1 ± 0.1	0.08 ± 0.01	0.03 ± 0.01
	(B) UDPGlc			
SSIII-D123	6.37 ± 0.51	3.2 ± 0.8	0.07 ± 0.01	0.011 ± 0.002
SSIII-D23	6.71 ± 0.50	3.7 ± 0.9	0.08 ± 0.01	0.011 ± 0.002
SSIII-D3	6.60 ± 0.92	2.6 ± 0.8	0.08 ± 0.02	0.012 ± 0.004
SSIII-CD	6.69 ± 0.56	2.5 ± 0.4	0.06 ± 0.01	0.009 ± 0.002

When ADPGlc was used as the substrate, all the SSIII isoforms displayed Michaelis kinetics. The $S_{0.5}$ values for the acceptor polysaccharides were 0.26, 0.68, 1.92, and 2.65 mg/mL glycogen for SSIII-D123, SSIII-D23, SSIII-D3, and SSIII-CD, respectively (Table 2A). The saturation plots are shown in Figure 3A. These results revealed that the presence of the N-terminal SBDs increased the apparent affinity for glycogen. SSIII-D123 displayed a higher V_{max} (80-fold) than SSIII-CD (Figure 3A). Furthermore, the full-length enzyme exhibited the highest catalytic efficiency as compared to the truncated forms [770-fold higher than that of SSIII-CD (Table 2A)]. Thus, the full-length enzyme SSIII-D123, containing the three N-terminal SBDs, exhibited a 10-fold decrease in the $S_{0.5}$ for glycogen and a nearly 80-fold increase in specific activity with respect to the isoform lacking these domains (SSIII-CD). Moreover, SSIII-D123 showed the highest catalytic efficiency compared with the truncated isoforms in the presence of ADPGlc. In contrast, when UDPGlc was used as the substrate, all the enzymes display cooperative kinetics (Figure 3B), and no changes in the apparent affinity for glycogen, V_{max}, or catalytic efficiency were observed with any SSIII isoforms (Table 2B and Figure 3B).

Effect of SBDs on the Kinetic Parameters of Starch Synthase III for ADPGlc and UDPGlc. Table 3 lists the kinetic parameters for the different starch synthase III isoforms using ADPGlc or UDPGlc as the sugar donor substrate. With ADPGlc, SSIII-D123 and SSIII-D23 dis-

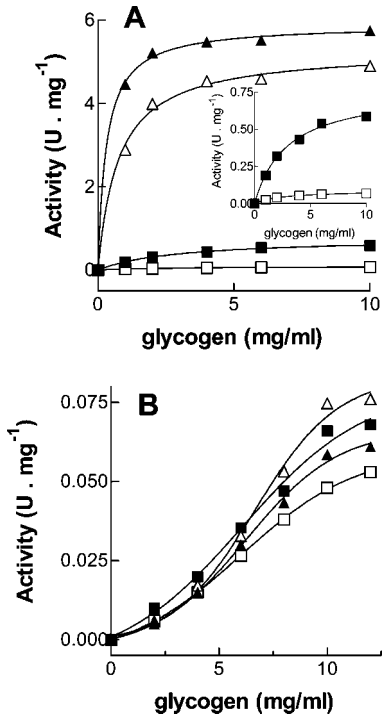

FIGURE 3: Glycogen saturation plots for SSIII enzymes performed in the presence of ADPGlc (A) or UDPGlc (B): SSIII-D123 (▲), SSIII-D23 (△), SSIII-D3 (■), and SSIII-CD (□). The inset in panel A displays a zoom of the plots for SSIII-D3 and SSIII-CD.

played higher V_{max} values (5.75 and 5.10 units/mg, respectively), whereas SSIII-D3 displayed an 10-fold decrease in V_{max}. SSIII-CD, lacking the three SBDs, has a V_{max} value 80-fold lower than that of the SSIII-D123 enzyme (Table 3). Furthermore, we determined the $S_{0.5}$ values for ADPGlc in the presence of saturating concentrations of glycogen. Although the SSIII-CD isoform displays the lowest $S_{0.5}$ value for ADPGlc (0.24 mM), the full-length enzyme has an $S_{0.5}$ value that is 18-fold higher. The apparent affinity for ADPGlc of the different isoforms ranged from 0.24 (for SSIII-CD) to 4.28 mM (for SSIII-D123) (Table 3). These results indicate that the increase in $S_{0.5}$ and V_{max} when the donor substrate is ADPGlc correlates with the number of N-terminal SBDs.

As described above, all SSIII isoforms exhibited activity also with UDPGlc; however, in the presence of UDPGlc, all the isoforms exhibited a similar $S_{0.5}$ value (2.5 mM) (Table 3) and a small change in V_{max} of <2-fold variation, in comparison to the 80-fold change in V_{max} when ADPGlc was the substrate (Table 3). These results indicate that the presence of SBDs had a weaker effect on the kinetic parameters when UDPGlc was the substrate.

Panels A and B of Figure 4 show the saturation plots of SSIII-D123 and SSIII-CD, respectively, with ADPGlc or UDPGlc. One can see that the full-length enzyme displays, in the presence of ADPGlc, a V_{max} that is 95-fold higher but an only 2-fold increase in apparent affinity compared to the values observed when UDPGlc is present (Figure 4A and Table 3), whereas SSIII-CD showed a small change in V_{max} but an 11-fold decrease in $S_{0.5}$ in the presence of ADPGlc with respect to the $S_{0.5}$ of UDPGlc (Figure 4B and Table 3).

Binding Assays. We tested the adsorption of the different SBD recombinant proteins to raw starch at various protein concentrations. Figure 5 shows the adsorption isotherms for the binding of D123, D23, D2, D3, and the catalytic domain, SSIII-CD, to the polysaccharide. The amount of protein bound correlates with the increase in the initial protein concentration and the number of SBD modules. Thus, D3 and SSIII-CD proteins bind starch with low affinity (K_{ad} = 2.1 ± 0.3 and 1.7 ± 0.2, respectively), whereas D23 shows a significant increment in affinity (K_{ad} = 18.9 ± 1.2). Finally, the entire N-terminal domain, containing three SBD modules (D123), displays an only 10% higher affinity with respect to that of D23 (K_{ad} = 22.0 ± 1.8) (Figure 5). These data indicate that D3 has a low affinity, similar to that of the catalytic domain; however, the presence of the D2 domain (D23 protein) increases 10-fold the affinity for starch. To further investigate the contribution of the D2 domain, we assayed the binding of this domain to starch. Results show an increment of 6-fold in the level of binding to starch of recombinant D2 with respect to D3 (K_{ad} = 11.8 ± 1.5).

DISCUSSION

In vivo studies showed that the deficiency of SSIII leads to different alterations in amylopectin structure, chain length, or a starch excess phenotype. Thus, it has been proposed that the SSIII isoform has a regulatory role in starch synthesis (*6, 17, 20*). However, there are only few studies on the biochemical characterization of this enzyme (*9, 11, 19, 21*).

We had previously described that *A. thaliana* SSIII has three internal repeats in its N-terminus which encode SBDs and that the C-terminal region contains the catalytic domain (*11, 19*). Indeed, we had proposed a structural model of SSIII-CD that predicts a global structural similarity of SSIII-CD and *Ag. tumefaciens* glycogen synthase (*19*). In this work, we provide experimental evidence of the role of the N-terminal starch-binding domains and its effect on the catalytic properties of SSIII.

SBDs are noncatalytic modules that belong to the carbohydrate-binding module (CBM) family known to facilitate the binding (and degradation) of starch (*12, 13*). Initially, only N- and C-terminal types of SBDs had been described; however, more recently, other modules playing a role as a SBD have also been reported (*12, 13*). In general, these modules comprise 130 residues (*22*). On the basis of sequence analysis, SBDs have been described in carbohydrate-binding module families CMB20, -21, -25, -26, -34, -41, -48, and -45 (*12, 13, 22*). Recently, the SBDs from SSIII have been included in the CBM21 family (*13*). To evaluate the role of these N-terminal SBDs in SSIII activity, we constructed truncated isoforms of the enzyme lacking one, two, or three SBDs and compared them with the full-length recombinant SSIII. Furthermore, SDBs proteins were generated for analysis of the specific function of these modules.

Kinetic experiments showed that SSIII-CD exhibited the highest apparent affinity for ADPGlc. However, the presence of one, two, or three SBDs reduced the apparent affinities for this sugar nucleotide. Under ADPGlc saturating conditions, we observed that the presence of the different SBDs resulted in a lower $S_{0.5}$ value for glycogen. Furthermore, there is a sequential increment in V_{max} depending on the number of SBDs, the highest V_{max} value corresponding to SSIII-D123 (Table 2A). Previously, we reported that the catalytic domain, SSIII-CD, is able to use ADPGlc or UDPGlc as the substrate. In contrast to the results obtained with ADPGlc, the apparent affinity for UDPGlc was similar for all the proteins without

Table 3: Kinetic Parameters for the Substrate ADPGlc or UDPGlc of SSIII Proteins

	ADPGlc			UDPGlc		
isoform	$S_{0.5}$ (mM)	n_H	V_{max} (units/mg)	$S_{0.5}$ (mM)	n_H	V_{max} (units/mg)
SSIII-D123	4.28 ± 0.68	1.2 ± 0.5	5.75 ± 0.78	2.49 ± 0.91	0.9 ± 0.2	0.06 ± 0.01
SSIII-D23	2.78 ± 0.41	2.6 ± 0.8	5.10 ± 0.73	2.77 ± 0.64	3.1 ± 1.8	0.08 ± 0.02
SSIII-D3	1.89 ± 0.10	1.5 ± 0.3	0.61 ± 0.04	2.44 ± 0.30	1.6 ± 0.3	0.07 ± 0.03
SSIII-CD	0.24 ± 0.02	0.8 ± 0.3	0.07 ± 0.02	2.62 ± 0.40	2.6 ± 0.9	0.05 ± 0.01

major changes in V_{max} (Table 2B). Indeed, no change in the UDPGlc apparent affinity or V_{max} was observed with any of the SSIII isoforms. We reported that the amino acid residues of SSIII participating in the binding of AGPGlc are evolutionarily conserved (*19*). However, the nonconservation of the Gly-X-Gly motif strictly conserved in the nucleotide binding site of all GT5 ADPGlc binding enzymes could explain the sugar nucleotide promiscuity as it was described for archea GSs (*19, 21*).

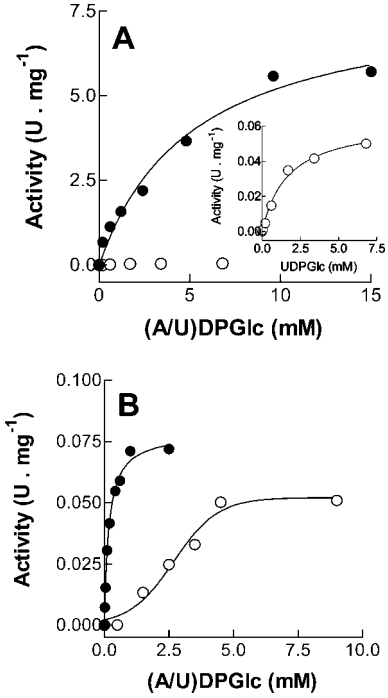

FIGURE 4: ADPGlc (●) or UDPGlc (○) saturation plots for SSIII-D123 (A) and SSIII-CD (B) enzymes determined in the presence of glycogen (12 mg/mL).

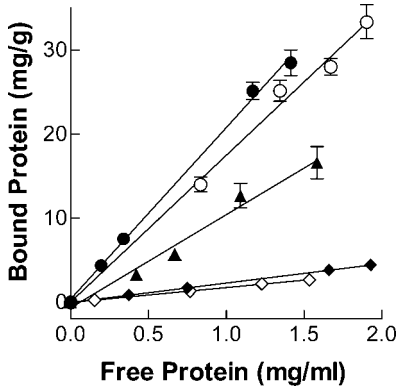

FIGURE 5: Adsorption of purified SBD proteins to cornstarch: D123 (●), D23 (○), D2 (▲), D3 (◆), and SSIII-CD (◇). Linear adsorption isotherms indicate the apparent equilibrium distribution of SBD proteins between the solid (bound protein) and liquid phase (free protein) at various protein concentrations. K_{ad} (milliliters per gram of starch) values represent the slopes of each isotherm.

The increment of the apparent affinity for glycogen in the presence of ADPGlc could be correlated with the nature of the SBD module that binds polysaccharides. This also suggests that the SBD modules have a regulatory role and are necessary for optimal catalysis of the full-length enzyme. The SSIII-D123 isoform has a lower $S_{0.5}$ value for UDPGlc; however, in the presence of ADPGlc, this protein exhibits an 95-fold increase in V_{max}. This is in agreement with the preference of using ADPGlc as the donor substrate of almost all the bacterial and plants glycogen (starch) synthases (*3*).

We have also determined the starch binding capacity of the different SBDs proteins, containing one, two, or three SBD modules. The comparison of the K_{ad} values showed that the D2 protein has an approximately 6-fold higher binding affinity than D3; however, the D23 protein exhibited an increase of 10-fold in its affinity for starch. Thus, the D23 protein makes the greatest contribution in the adsorption of the polysaccharide. Our results indicate that each peptide does not contribute equally to the adsorption of starch and that the second and third SBDs in tandem act synergistically rather than additively in starch binding. These data are in agreement with those reported by Guillen et al. (*23*) which described a cooperative effect of tandemly arranged SBDs from a *Lactobacillus amylovorus* α amylase on starch binding. Similar results were reported for the CBMs of cellulases and xylanase-degrading enzymes (*24, 25*) but not for synthesizing enzymes such as SSIII. The origin of multiple binding modules remains unclear (*23*). The presence of tandem modules in starch synthase III could be an evolutionary adaptation designed to allow the synthesis and/or restructuring of starch, allowing the fixation of the enzyme over the substrate.

In summary, our results support the importance of the N-terminal SBDs, particularly the D23 domain, in the binding of starch and in the regulation and catalysis of SSIII. This is the first experimental report that characterizes the role and function of SBDs in a synthesizing enzyme. This work also provides the foundation for future work aimed at elucidating the functions of N- and C-termini and in tandem SBDs present in different proteins. The availability of other SBDs such as that from the glucoamylase from *As. niger* (*26*) and other recombinant starch (glycogen) synthases may lead to generation of chimeric enzymes for further analysis of the functions of SBDs. Indeed, the data presented here may contribute to a better understanding of the synthesis of the starch granule and to an improvement in our knowledge of the functional role of starch-binding domains in plants.

EXPERIMENTAL PROCEDURES

Plant Material. A. thaliana (var. Columbia Col-0) plants grown in a greenhouse were used in these experiments.

Strains and Culture Media. E. coli XL1Blue {*endA*1, *gyrA*46, *hsdR*17, *lac*$^-$, *recA*1, *relA*1, *supE*44, *thi*-1, F′

[*proAB*$^+$, *lacI*^{q} *lacZ*ΔM15, Tn*10*(tet^r)]} and *E. coli* BL21-CodonPlus(DE3)-RIL [*E. coli* B F- *ompT hsdS*(rB- mB-) *dcm*+ Tetr *gal* (DE3) *endA* Hte [*argU ileY leuW* Cam^r]] were used in this study. *E. coli* strains were grown at 37 °C in LB medium containing the appropriate antibiotics.

Molecular Cloning of SSIII, Isoforms, and SBD Proteins from A. thaliana. Total RNA was isolated from *A. thaliana* leaves using Tri reagent (Sigma-Aldrich, St. Louis, MO). This sample was used as a template for cDNA synthesis using oligo(dT) primer and the MVLV RT-PCR system (USB Corp., Cleveland, OH). cDNA corresponding to *SSIII* (At1g11720) was PCR amplified using *Pfu* polymerase (Promega Corp., Madison, WI) and the following primers (5′ primers): 123up, AGAGCATATGAGTGCTCAGAAAAGA (*Nde*I site underlined); 23up, AGAGCATATGATTAAGAAAGCTGTA (*Nde*I site underlined); 3up, AGAGCATATGAGAAAACTTCAGGAG (*Nde*I site underlined); and cdup, AGAGCATATGCACATTGTTCAT (*Nde*I site underlined). SSIIIdo was used as a 3′ primer for the amplification of SSIII enzymes: AAACTCGAGCTTGCGTGCAGAGTG (*Xho*I site underlined). SBD proteins were obtained using primer 123up, 23up, or 3up, combined with SBDdo [CTGCTCGAGTGGTTCCTTTGAAAT (*Xho*I site underlined)]. D2 was obtained using 23up and D2do primer [TTTCTCGAGTCTTTCCTTAGTTTCAGC (*Xho*I site underlined)]. The resulting PCR products were digested using restriction endonucleases *Nde*I and *Xho*I and cloned into the pET32c vector (Novagen Inc., Madison, WI). XL1Blue *E. coli* cells were transformed with each construction. Positive clones were verified by DNA sequencing using the BigDye-Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The new vectors encode the following: pVAL15, full-length SSIII (SSIII-D123, containing the three SBDs, 3036 bp); pVAL17, SSIII-D23 (containing SBD2 and SBD3, 2232 bp); pVAL18, SSIII-D3 (containing only one SBD, SBD3, 1734 bp); pVAL3, SSIII-CD (containing the catalytic C-terminal domain of SSIII, 1374 bp); pVAL19, D123 (containing the three N-terminal SBDs, 1662 bp); pVAL20, D23 (containing SBDs 2 and 3, 858 bp); pVAL21, D3 (the third SBD, 360 bp); pVAL25, D2 (498 bp). Each vector was used to transform BL21-CodonPlus (DE3)-RIL *E. coli* competent cells for the purpose of expression.

Expression and Purification of SSIII Enzymes and SBDs Proteins. Recombinant proteins were expressed in *E. coli* BL21(DE3)-RIL cells. After the cells had been grown at 37 °C for 4 h, 0.5 mM IPTG was added to the culture medium and incubated at 28 °C for at least 4 h. Cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C. Each pellet was washed and suspended in buffer containing 20 mM Tris-HCl (pH 8) (1 mL/g of cells). Cells were disrupted using an ultrasonicator (VCX130, Sonics and Materials Inc.) and centrifuged at 12000*g* for 15 min at 4 °C. The homogenates were filtered through a 0.2 μm cellulose acetate membrane filter and loaded onto a HiTrap chelating HP column (Amersham Biosciences) equilibrated with binding buffer [20 mM Tris-HCl (pH 8), 0.3 M NaCl, 1 mM β-mercaptoethanol, and 20 mM imidazole]. This step was performed at 25 °C. The column was washed with at least 10−15 volumes of binding buffer, and each protein was eluted using a linear gradient of binding buffer and elution buffer [20 mM Tris-HCl (pH 8), 0.3 M NaCl, 1 mM β-mercaptoethanol, and 0–500 mM imidazole]. All the proteins eluted in the fraction between 200 and 300 mM imidazole. The active fractions were pooled and concentrated to >1 mg/mL using Centriplus-10 and Centriprep-10 concentrators (Millipore Corp.). The concentrated proteins were desalted and used to determine enzyme activity or stored at −20 °C until they were used. The presence of the different forms of SSIII or SBDs was monitored in chromatography fractions by measurement of their enzymatic activity (in the case of SSIII isoforms), SDS−PAGE, and immunoblotting.

Binding Assays. Adsorption of SBD to raw starch was measured as described previously with minor modifications (*23*). Purified recombinant SBD proteins (final concentration of 0−80 μM) were added to a prewashed raw cornstarch suspension (10%, w/v) in 100 mM citrate-phosphate buffer (pH 5) to a final volume of 60 μL. Each mixture was incubated at 4 °C for at least 1 h with gentle shaking (10 rpm) and centrifuged at 12000*g* for 5 min at 4 °C. To calculate the amount of bound protein, the amount of protein in the supernatant was subtracted from the total protein added to the mixture. The protein concentration was determined by measuring the absorbance at 280 nm. The adsorption constant (K_{ad}, in milliliters per gram of starch) was determined from the slope as previously reported (*27*).

Determination of Starch Synthase Activity. The activity of *A. thaliana* SSIII was determined using a radiochemical method previously described with some modifications (*28*). Assays were initiated by the addition of the enzyme and also were performed at different enzyme concentrations to ensure steady-state conditions. The assay medium contained 100 mM MOPS (pH 7.5), 0.25 mM KCl, 10 mg of rabbit muscle glycogen (Sigma-Aldrich) or amylopectin (Fluka, Sigma-Aldrich), and 1.25 mM ADP[^{14}C]Glc (1.3 μCi/nmol) or UDP[^{14}C]Glc (1.1 μCi/nmol) as the glycosyl donor. The final volume of each reaction was 100 μL. After 20 min at 30 °C, reactions were stopped by the addition of 500 μL of 75% (v/v) methanol and 1% (w/v) KCl, mixtures centrifuged, and pellets washed twice with the same solution. Radioactivity incorporated in the final pellet suspended in 200 μL of distilled water was determined in a liquid scintillation counter. One unit of activity is defined as the amount of enzyme catalyzing the incorporation into glycogen of 1 μmol of [^{14}C]Glc (from ADPGlc or UDPGlc) per minute at 30 °C.

Protein Measurements. The total amount of protein was determined using the Bradford method as described previously (*29*).

Gel Electrophoresis and Immunological Studies. Denaturing gel electrophoresis was performed with the Bio-Rad Mini Protean system using 10% polyacrylamide/bisacrylamide gels as described by Laemmli (*30*). Gels were revealed by staining with Coomassie Brilliant Blue (R250, Sigma-Aldrich) or electroblotted onto nitrocellulose membranes (Trans-Blot Transfer Medium, Nitrocellulose Membrane, Bio-Rad, Hercules, CA). After being electroblotted, membranes were treated with penta-His antibody (anti-His antibody selector kit, Qiagen) or polyclonal antibodies raised against recombinant *Ag. tumefaciens* glycogen synthase as described previously (*28*). The antigen–antibody complex was visualized with alkaline phosphatase-linked anti-mouse IgG or anti-rabbit IgG, followed by staining with BCIP and NBT as described previously (*31*).

ACKNOWLEDGMENT

We are grateful to Jose Luis Burgos (CIC) for excellent technical assistance.

REFERENCES

1. Ball, S., Guan, H. P., James, M., Myers, A., Keeling, P., Mouille, G., Buleon, A., Colonna, P., and Preiss, J. (1996) From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. *Cell 86*, 349–352.
2. Martin, C., and Smith, A. M. (1995) Starch biosynthesis. *Plant Cell 7*, 971–985.
3. Ball, S. G., and Morell, M. K. (2003) From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. *Annu. Rev. Plant Biol. 54*, 207–233.
4. Delvalle, D., Dumez, S., Wattebled, F., Roldan, I., Planchot, V., Berbezy, P., Colonna, P., Vyas, D., Chatterjee, M., Ball, S., Merida, A., and D'Hulst, C. (2005) Soluble starch synthase I: A major determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. *Plant J. 43*, 398–412.
5. Maddelein, M. L., Libessart, N., Bellanger, F., Delrue, B., D'Hulst, C., Van den Koornhuyse, N., Fontaine, T., Wieruszeski, J. M., Decq, A., and Ball, S. (1994) Toward an understanding of the biogenesis of the starch granule. Determination of granule-bound and soluble starch synthase functions in amylopectin synthesis. *J. Biol. Chem. 269*, 25150–25157.
6. Zhang, X., Myers, A. M., and James, M. G. (2005) Mutations affecting starch synthase III in *Arabidopsis* alter leaf starch structure and increase the rate of starch synthesis. *Plant Physiol. 138*, 663–674.
7. Delrue, B., Fontaine, T., Routier, F., Decq, A., Wieruszeski, J. M., Van Den Koornhuyse, N., Maddelein, M. L., Fournet, B., and Ball, S. (1992) Waxy *Chlamydomonas reinhardtii*: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *J. Bacteriol. 174*, 3612–3620.
8. Roldan, I., Wattebled, F., Mercedes Lucas, M., Delvalle, D., Planchot, V., Jimenez, S., Perez, R., Ball, S., D'Hulst, C., and Merida, A. (2007) The phenotype of soluble starch synthase IV defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation enzymes in the control of starch granule formation. *Plant J. 49*, 492–504.
9. Senoura, T., Asao, A., Takashima, Y., Isono, N., Hamada, S., Ito, H., and Matsui, H. (2007) Enzymatic characterization of starch synthase III from kidney bean (*Phaseolus vulgaris* L.). *FEBS J. 274*, 4550–4560.
10. Li, Z., Mouille, G., Kosar-Hashemi, B., Rahman, S., Clarke, B., Gale, K. R., Appels, R., and Morell, M. K. (2000) The structure and expression of the wheat starch synthase III gene. Motifs in the expressed gene define the lineage of the starch synthase III gene family. *Plant Physiol. 123*, 613–624.
11. Palopoli, N., Busi, M. V., Fornasari, M. S., Gomez-Casati, D., Ugalde, R., and Parisi, G. (2006) Starch-synthase III family encodes a tandem of three starch-binding domains. *Proteins 65*, 27–31.
12. Machovic, M., and Janecek, S. (2006) Starch-binding domains in the post-genome era. *Cell. Mol. Life Sci. 63*, 2710–2724.
13. Machovic, M., and Janecek, S. (2006) The evolution of putative starch-binding domains. *FEBS Lett. 580*, 6349–6356.
14. Bibel, M., Brettl, C., Gosslar, U., Kriegshauser, G., and Liebl, W. (1998) Isolation and analysis of genes for amylolytic enzymes of the hyperthermophilic bacterium *Thermotoga maritima*. *FEMS Microbiol. Lett. 158*, 9–15.
15. Abe, A., Tonozuka, T., Sakano, Y., and Kamitori, S. (2004) Complex structures of *Thermoactinomyces vulgaris* R-47 α-amylase 1 with malto-oligosaccharides demonstrate the role of domain N acting as a starch-binding domain. *J. Mol. Biol. 335*, 811–822.
16. Janecek, S. (2002) A motif of a microbial starch-binding domain found in human genethonin. *Bioinformatics 18*, 1534–1537.
17. Gao, M., Wanat, J., Stinard, P. S., James, M. G., and Myers, A. M. (1998) Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell 10*, 399–412.
18. Dian, W., Jiang, H., and Wu, P. (2005) Evolution and expression analysis of starch synthase III and IV in rice. *J. Exp. Bot. 56*, 623–632.
19. Busi, M. V., Palopoli, N., Valdez, H. A., Fornasari, M. S., Wayllace, N. Z., Gomez-Casati, D., Parisi, G., and Ugalde, R. (2007) Functional and structural characterization of the catalytic domain of the starch synthase III from *Arabidopsis thaliana*. *Proteins 70*, 31–40.
20. Edwards, A., Fulton, D. C., Hylton, C., Jobling, S. A., Gidley, M., Rössner, U., Martin, C., and Smith, A. (1999) A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. *Plant J. 17*, 251–261.
21. Horcajada, C., Guinovart, J. J., Fita, I., and Ferrer, J. C. (2006) Crystal structure of an archaeal glycogen synthase: Insights into oligomerization and substrate binding of eukaryotic glycogen synthases. *J. Biol. Chem. 281*, 2923–2931.
22. Rodriguez-Sanoja, R., Oviedo, N., and Sanchez, S. (2005) Microbial starch-binding domain. *Curr. Opin. Microbiol. 8*, 260–267.
23. Guillen, D., Santiago, M., Linares, L., Perez, R., Morlon, J., Ruiz, B., Sanchez, S., and Rodriguez-Sanoja, R. (2007) α-Amylase starch binding domains: Cooperative effects of binding to starch granules of multiple tandemly arranged domains. *Appl. Environ. Microbiol. 73*, 3833–3837.
24. Boraston, A. B., McLean, B. W., Chen, G., Li, A., Warren, R. A., and Kilburn, D. G. (2002) Co-operative binding of triplicate carbohydrate-binding modules from a thermophilic xylanase. *Mol. Microbiol. 43*, 187–194.
25. Bolam, D. N., Xie, H., White, P., Simpson, P. J., Hancock, S. M., Williamson, M. P., and Gilbert, H. J. (2001) Evidence for synergy between family 2b carbohydrate binding modules in *Cellulomonas fimi* xylanase 11A. *Biochemistry 40*, 2468–2477.
26. Sorimachi, K., Jacks, A. J., Le Gal-Coeffet, M. F., Williamson, G., Archer, D. B., and Williamson, M. P. (1996) Solution structure of the granular starch binding domain of glucoamylase from *Aspergillus niger* by nuclear magnetic resonance spectroscopy. *J. Mol. Biol. 259*, 970–987.
27. Rodriguez-Sanoja, R., Ruiz, B., Guyot, J. P., and Sanchez, S. (2005) Starch-binding domain affects catalysis in two *Lactobacillus* α-amylases. *Appl. Environ. Microbiol. 71*, 297–302.
28. Ugalde, J. E., Parodi, A. J., and Ugalde, R. A. (2003) De novo synthesis of bacterial glycogen: *Agrobacteriumtumefaciens* glycogen synthase is involved in glucan initiation and elongation. *Proc. Natl. Acad. Sci. U.S.A. 100*, 10659–10663.
29. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.
30. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
31. Bollag, D. M., Rozycki, M. D., and Edelstein, S. J. (1996) *Protein methods*, 2nd ed., Vol. 415, Wiley-Liss, New York.

BI702418H