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ARTICLE *in* BIOCHEMISTRY · AUGUST 2000

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Involvement of Conserved Aspartate and Glutamate Residues in the Catalysis and Substrate Binding of Maize Starch Synthase‡

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Received February 22, 2000; Revised Manuscript Received May 1, 2000

ABSTRACT: Chemical modification of maize starch synthase IIb-2 (SSIIb-2) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), which modifies acidic amino acid residues, resulted in a time- and concentration-dependent inactivation of SSIIb-2. ADPGlc was found to completely protect SSIIb-2 from inactivation by EDAC. These results suggest that glutamate or aspartate is important for SS activity. On the basis of the sequence identity of SS, conserved acidic amino acids were mutagenized to identify the specific amino acid residues important for SS activity. Three amino acids (D21, D139, and E391) were found to be important for SS activity. D21N showed 4% of the wild-type enzyme activity and a 10-fold decrease in the affinity for ADPGlc, while the conservative change from D21 to E resulted in a decrease in V_{max} and no change in affinity for ADPGlc, suggesting that the negative charge is important for ADPGlc binding. When sites D139 and E391 were changed to their respective amide form, no SS activity was detected. With the conservative change, D139E showed a decrease in V_{max} and no changes in apparent K_m for substrates. E391D showed a 9-fold increase in K_m for ADPGlc, a 12-fold increase in apparent K_m for glycogen, and a 4-fold increase in apparent K_m for amylopectin. The circular dichroism analysis indicates that these kinetic changes may not be due to a major conformation change in the protein. These results provide the first evidence that the conserved aspartate and glutamate residues could be involved in the catalysis or substrate binding of SS.

Starch is a major storage compound in higher plants. With increasing demand for starch as food, feed, and industrial raw materials, a better understanding of starch biosynthesis would provide a sound biotechnological strategy to improve starch quality and quantity. At least four major enzymes are involved in the starch synthesis: ADPGlc pyrophosphorylase (AGPase[1]), starch synthase (SS), branching enzyme (BE), and debranching enzyme (DBE). AGPase catalyzes the reaction forming ADPGlc from glucose-1-phosphate and ATP. SS catalyzes the elongation of α-(1,4) glucans by adding glucose units from ADPGlc to the nonreducing end of the growing chain. BE introduces α-(1,6) linkages in starch by cleaving α-(1,4) linkages and simultaneously forming the α-(1,6) linkages, while DBE catalyzes the cleavage of α-(1,6) linkages. Biochemical and genetic studies have shown that maize SS plays an important role in determining starch quantity (*1*) and quality (*2*).

There are multiple forms of SS in maize endosperm. The genes which code for GBSS, SSI, SSIIa, SSIIb, and *dull*1 have been cloned and characterized (*2*−*5*). Maize SSI, SSIIa, and SSIIb have been shown to possess distinct kinetic properties (*6*, *7*). For example, SSI and SSIIb have a higher V_{max} with glycogen as primer while SSIIa has a higher V_{max} with amylopectin as primer. SSIIb exhibits a specific activity 2−3-fold higher than SSI and SSIIa (*6*, *7*). Structurally, all soluble SS enzymes have a conserved C-terminus and a divergent N-terminal extension, with the catalytic domain being located in the C-terminus (*6*, *7*). The N-terminally truncated SSIIb (SSIIb-2) is similar in size (489 aa) to GBSS (533 aa) and the *Escherichia coli* glycogen synthase (477 aa). The truncated SSIIb-2 was chosen for this study because it has catalytic properties similar to the full-length SSIIb and is much easier to purify (*7*).

Apart from a few studies on *E. coli* glycogen synthase and maize SSIIa, little is known about the structure−function relationship of starch synthase. Studies of *E. coli* glycogen synthase using affinity labeling with adenosine diphosphopyridoxal and site-directed mutagenesis have suggested that the lysine residue in the conserved KTGGL sequence is involved in ADPGlc binding (*8*, *9*). Work performed in our lab indicated essential arginine residues may be important for substrate binding of SSIIa (*10*). Previous studies have shown that aspartate and glutamate residues are involved in catalysis of amylolytic enzymes such as BE and α-amylase (*11*−*16*). Because BE and SS can use the same substrate,

‡ This paper is part of the Master's Degree thesis for Deborah J. Nichols at Iowa State University.

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[1] Abbreviations: ADPGlc, adenosine diphosphoglucose; AGPase, ADPGlc pyrophosphorylase; BE, branching enzyme; CD, circular dichroism; DBE, debranching enzyme; dp, degree of polymerization; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GBSS, granule-bound starch synthase; MES, 2-[*N*-morpholino]ethanesulfonic acid; SS, starch synthase.

10.1021/bi000407g CCC: $19.00
Published on Web 06/09/2000

we decided to investigate the possible role of acidic amino acids in catalysis of starch synthase.

MATERIALS AND METHODS

[U-^{14}C]ADPGlc was synthesized from [U-^{14}C]glucose-1-phosphate (Amersham) and AGPase essentially as described in (*17*). All other supplies and chemicals were either from Sigma or as indicated.

EDAC Modification of SSIIb-2. Chemical modification of maize SSIIb-2 was performed as previously described (*11, 18*). The chemical modification reaction containing 4 μg of SSIIb, 25 mM phenylethylamine, and various concentrations of EDAC in 0.1 M MES (pH 6.5) was carried out at 30 °C. The reaction was stopped by removing 5 μL aliquots at different time points and diluting it 1/20 in 20 mM Tris-acetate, pH 8.0, 1 mM EDTA, and 5 mM DTT.

Site-Directed Mutagenesis of SSIIb-2. Site-directed mutagenesis was performed using PCR and following the directions from a kit from Stratagene. The primers listed below, along with the reverse complements, were made in varying lengths from 20 to 38 bases with a T_m of 66.9−78.4 °C. Changed base pairs are shown in lower case. To ensure that no other mutations had been introduced by PCR, a portion of the DNA containing the desired mutation was subcloned back into the wild-type plasmid and sequenced again.

mutant	primer	sequence
E9Q	ExS82	5′-GGTGGCTTCTcAATGTGCTCC-3′
D21N	ExS84	5′-GGCCTTGGAaATGTCGTGGG-3′
D21E	ExS169	5′-GTGGCCTTGGAGAaGTCGTGGGTGCTTTG-3′
D21K	ExS171	5′-GGTGGCCTTGGAaAaGTCGTGGGTGCTTTGC-3′
E116Q	ExS86	5′-GCCGCTGTTcAGGTTCCATGG-3′
D139N	ExS136	5′-GTTTTCATTGCTAATaATTGGCATACCGCAC-3′
D139E	ExS175	5′-GTTTTCATTGCTAATGAaTGGCATACCGCAC-3′
D139K	ExS177	5′-TTTTCATTGCTAATaAaTGGCATACCGCACTTCT-GCC-3′
D217N	ExS88	5′-GAAGACGGCAaACCGGGTGGTG-3′
E229Q	ExS138	5′-GGCTACATGTGGcAGCTGAAGACTTCG-3′
D258N	ExS173	5′-CGTGAACGGCATCaACATGAGCGAGTGG-3′
D267N	ExS92	5′-CCCGCTGTGaACGTGCACCTC-3′
D322N	ExS94	5′-GAAGGGCGTGaACATCATCGCC-3′
D382N	ExS140	5′-GGCGGGCGCGaACATCCTGCTGATG-3′
E391Q	ExS142	5′-GTCGCGGTTCcAGCCGTGCGGG-3′
E391D	ExS179	5′-GTCGCGGTTCGAcCCGTGCGGG-3′
E391K	ExS181	5′-CCGTCGCGGTTCaAaCCGTGCGGGC-3′
D417N	ExS96	5′-GGGCTCCGGaACACGGTGGC-3′

Expression, Purification, and Activity Assays of SSIIb-2. Expression, purification, and SS assays of SSIIb-2 were performed as previously described (*6, 7*). The purified enzymes were stored in the presence of 20% glycerol at −70 °C. SS assays were performed using either 5 mg/mL amylopectin or 20 mg/mL glycogen. Kinetics were performed in duplicate at two time points, 8 and 13 min, to make sure the reaction was linear at each concentration of ADPGlc or primer. After the reaction was stopped by heating, the carrier glycogen (5 μL; 100 mg/mL) was added and the unreacted [U-^{14}C]ADPGlc was removed by precipitating the glucans with 75% methanol/1% KCl (*19*).

RESULTS

Chemical Modification of SSIIb-2 by EDAC. To study the possible role of acidic amino acids in SSII catalysis, chemical modification of SSIIb-2 by EDAC was performed. Inactivation by EDAC was time- and concentration-dependent,

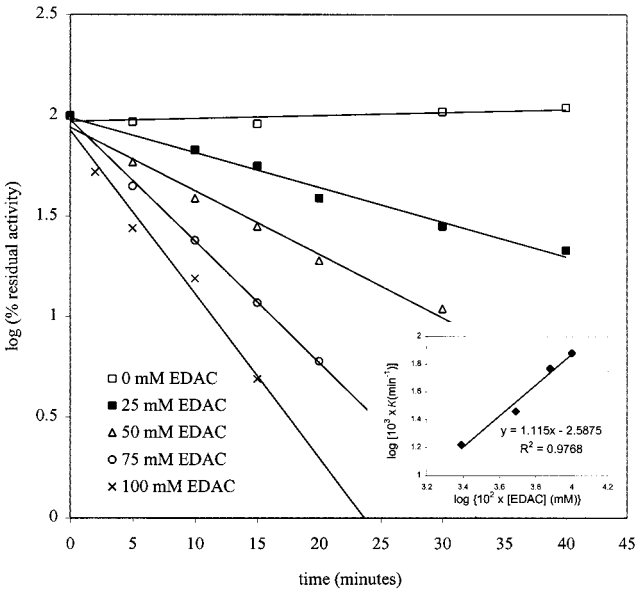

FIGURE 1: Chemical modification of SSIIb-2 by EDAC. SSIIb-2 was incubated with EDAC at 0 mM (□), 25 mM (■), 50 mM (△), 75 mM (○), and 100 mM (×). The slopes of the lines were used to determine pseudo-first-order rate constants (*k*). Inset: Double log plot of rate constants vs EDAC concentration. The resulting line with a slope of 1.1 suggests at least one acidic amino acid is important in catalysis.

resulting in a linear relationship between log % activity vs time, indicating pseudo-first-order rate kinetics (Figure 1). A log plot of inactivation rate constants, determined from the slopes of the lines in Figure 1 vs EDAC concentration, resulted in a straight line with a slope of 1.1 (Figure 1 inset). The slope 1.1 suggests that at least one acidic amino acid is important for SS activity. ADPGlc was found to protect SSIIb-2 from inactivation by EDAC. The ability of ADPGlc to protect SSIIb-2 was dependent on the concentration of ADPGlc (Figure 2). The double reciprocal plot of Δk, determined from the slopes of the lines in Figure 2, vs ADPGlc concentration resulted in a straight line (Figure 2 inset). Analysis of the line in Figure 2 inset shows that 0.2 mM ADPGlc is needed for half-maximal protection in the presence of 75 mM EDAC. This value is slightly higher than the K_m for ADPGlc (0.13 mM). In contrast, maltodextrin (dp 16.5−19.5; Aldrich Chemical Co.) only partially protected SSIIb-2 from EDAC inactivation (Figure 3). Increasing concentration of maltodextrin protected SSIIb-2 from EDAC inactivation up to 80%. An additional increase in the concentration of maltodextrin (up to 127.8 mg/mL) did not provide further protection from EDAC inactivation. A number of other substances, including amylopectin, glycogen, ATP, ADP, and glucose, were found not to protect SSIIb-2 from EDAC inactivation.

Site-Directed Mutagenesis of Conserved Aspartate and Glutamate Residues. Chemical modification by EDAC has suggested that at least one acidic amino acid may be involved in the catalysis or substrate binding of SSIIb. To identify specific amino acids important for SS activity, site-directed mutagenesis was used to generate SS mutants. Sequence alignment of SS genes indicates that four glutamate and eight aspartate residues are conserved among all maize SS isozymes and *E. coli* glycogen synthase. Therefore, mutants were generated individually for each of the 12 conserved acidic amino acids in SSIIb-2. Aspartate residues were

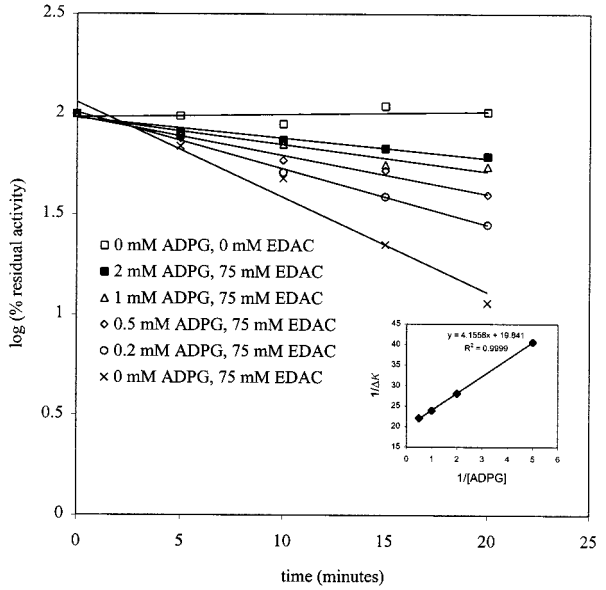

FIGURE 2: ADPGlc protection of SSIIb-2 from EDAC inactivation. SSIIb-2 was incubated with 0 mM EDAC and 0 mM ADPGlc (□). All other points contain 75 mM EDAC and different concentrations of ADPGlc: 2 mM (■), 1 mM (△), 0.5 mM (◇), 0.2 mM (○), and 0 mM (×). Inset: Double reciprocal plot of the change of the slopes (k) vs ADPGlc concentration. The reciprocal of the x-intercept shows that half-maximal protection by ADPGlc occurs at 0.2 mM.

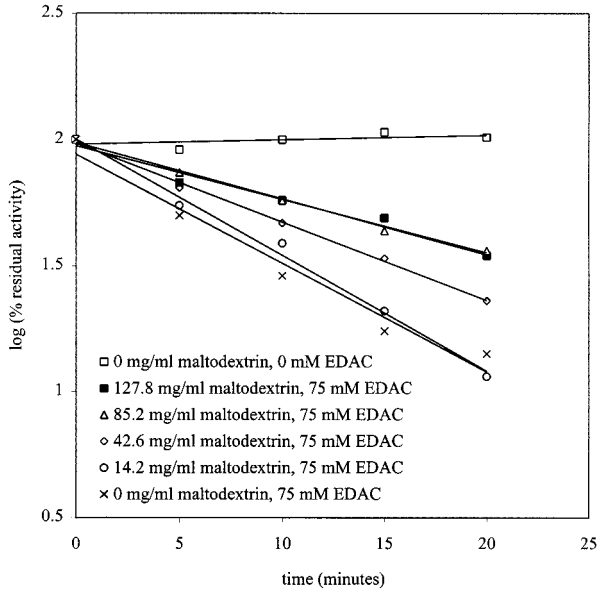

FIGURE 3: Maltodextrin (dp 16.5−19.5) partially protects SSIIb-2 from EDAC inactivation. SSIIb-2 was incubated with 0 mM EDAC and 0 mM maltodextrin (□). All other points contain 75 mM EDAC and different concentrations of maltodextrin: 127.8 mg/mL (■), 85.2 mg/mL (△), 42.6 mg/mL (◇), 14.2 mg/mL (○),and 0 mg/mL (×).

changed to asparagine, and glutamate residues were changed to glutamine.

The mutants were initially screened by assaying the specific activity in the crude *E. coli* extract. Western blot analysis has shown that all of the mutants and the wild-type enzyme were expressed at a similar level in *E. coli* (data not shown). Therefore, the specific activities of the mutants were compared to that of the wild-type enzyme. The percentage of activity is shown in Table 1. Four of the mutants (E229Q, D258N, D322N, and D417N) had 60−100% of the wild-type activity. Another four of the mutants (E116Q, D217N, D267N, and D382N) showed 17−31% of the wild-type SS activity. While mutant E9Q exhibited 9% of the wild-type enzyme activity, mutants D21N, D139N, and E391Q showed little or no SS activity. Because E9 is located very close to the K15 of the conserved KTGG motif (Figure 6), we determined the kinetic properties of E9Q in a crude extract. E9Q mutation did not affect its affinity for the substrates (data not shown) but did decrease its activity to 9% of that of the wild-type enzyme. This suggests that E9 is important for SS activity. Consequently, we focused our study on the other three sites. New mutants were generated for these three sites (D21, D139, and E391) by changing each site individually to lysine, or from aspartate to glutamate, or from glutamate to aspartate. These new mutants were screened in crude *E. coli* extract as above to determine the SS activity. While the mutants D21K, D139K, and E391K exhibited no detectable SS activity when the acidic amino acid was changed to a positive lysine residue, with a conservative change, D21E, D139E, and E391D individually showed 20%, 13%, and 10% of the wild-type enzyme activity, respectively. Wild-type SSIIb-2 and the active mutants at sites D21, D139, and E391 were then purified to apparent homogeneity as determined by SDS−PAGE gel electrophoresis.

Table 1: Activity of SSIIb-2 Mutants[a]

mutant	% wild-type activity in crude extract	mutant	% wild-type activity in crude extract
D258N	110	D382N	28
D322N	69	D267N	17
E229Q	61	E9Q	9
D417N	60	D21N	4
E116Q	31	D139N	0
D217N	25	E391Q	0

[a] Western blot analysis showed all of the mutants and the wild-type enzyme were expressed at a similar level in *E. coli*.

Kinetic Characterization of SSIIb-2 Mutants. Kinetic parameters for SSIIb-2 and mutants D21N, D21E, D139E, and E391D were carried out using either glycogen or amylopectin as primers in the presence of 0.5 M citrate (Table 2). All mutants showed a 70−96% decrease in V_{max} compared to the wild-type enzyme. Kinetic characterization of these mutants indicates that the acidic amino acids play a role in ADPGlc binding and catalysis. D21N showed a 10-fold increase in K_m (1.48 mM) for ADPGlc and did not reach saturation conditions of ADPGlc until 5 mM ADPGlc, while the wild-type enzyme was saturated at 1 mM with a K_m of 0.13 mM. However, mutant D21E conserved the negative charge and showed no change in K_m for ADPGlc, suggesting the negative charge is important in the binding of ADPGlc. Both D21E and D21N showed a 4−5-fold increase in apparent K_m for glycogen and a 1−3-fold increase in apparent K_m for amylopectin. E391D also exhibited a 9-fold increase in K_m for ADPGlc and did not reach saturation conditions of ADPGlc until 5 mM ADPGlc. Along with the decrease in affinity for ADPGlc, E391D exhibited a 13-fold increase in apparent K_m for glycogen and a 4-fold increase in apparent K_m for amylopectin. Interestingly, D139E showed only a 70% decrease in V_{max}, but no changes in apparent K_m for either substrate.

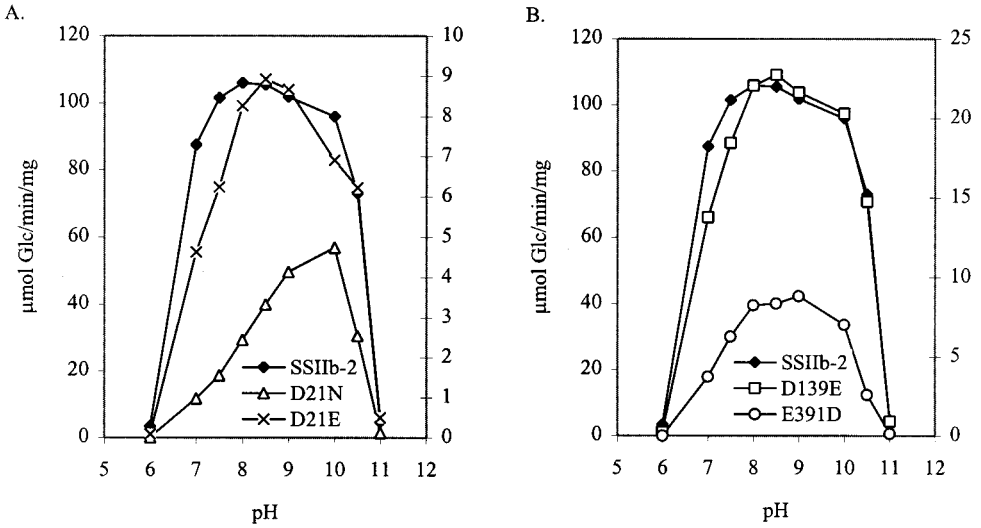

FIGURE 4: pH curves of SSIIb-2 mutants. (A) pH curves for WT SSIIb-2 (◆), D21E (×), and D21N (△). SSIIb-2 (◆) is plotted on the left *y*-axis; D21E (×) and D21N (△), on the right *y*-axis. (B) pH curve for WT SSIIb-2 (◆), D139E (□), and E391D (○). SSIIb-2 (◆) is plotted on the left *y*-axis; D139E (□) and E391D (○), on the right *y*-axis.

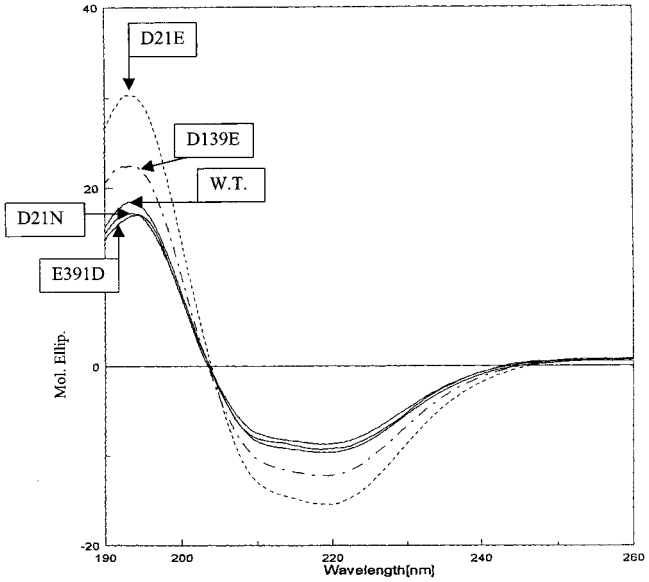

FIGURE 5: CD spectra of SSIIb-2 mutants.

	21	139	391
SSIIb-2	KTGGLG**D**V	FIAN**D**WHT	MPSRF**E**PCGLNQL
SSIIa-1	KTGGLG**D**V	FIAN**D**WHT	MPSRF**E**PCGLNQL
SSI	KSGGLG**D**V	FVVN**D**WHA	MPSRF**E**PCGLNQL
GBSS	KTGGLG**D**V	FVCN**D**WHT	VTSRF**E**PCGLIQL
E.coli GS	KTGGLG**D**V	VHAH**D**WHA	VPSRF**E**PCGLTQL

FIGURE 6: Sequence comparison of amino acid residues close to D21, D139, and E391. Sequences are taken from refs *4*, *5*, *20*, *21*. D21 is located close to the putative ADPGlc binding site in domain 1 (*9*).

pH Curves of SSIIb-2 Mutants. The wild-type SSIIb-2, D21E, D139E, and E391D showed a broad peak of activity between pH 7.0 and 10.0, with maximum activity occurring between pH 8 and 9 (Figure 4). In comparison with wild-type enzyme, the mutant D21N exhibited a narrower pH curve and shifted the optimum pH to 10.0. It appears that losing the carboxyl group in D21N increased its pK_a from 7 in wild-type enzyme to 8 in D21N, indicating the negative charge of D21 is important for catalysis.

Circular Dichroism (CD) Spectra of Purified SSIIb-2 Mutants. To determine whether the kinetic properties of the mutants are caused by a major conformational change in the protein, CD spectra of these enzymes were compared. After the purified protein samples were dialyzed into 10 mM sodium phosphate (pH 8.0), the CD measurements were performed by the Iowa State Protein Facility. No major changes were observed in the CD spectra between the mutants (D21N, D21E, D139E, and E391D) and wild-type SSIIb-2 (Figure 5).

DISCUSSION

Apart from a few studies on the lysine residues of *E. coli* glycogen synthase (*8*, *9*) and arginine residues of maize SSIIa (*10*), very little is known about the SS catalytic mechanism. Aspartate and glutamate have been found to be important in the catalysis of BE, α-amylases, and other amylolytic enzymes (*11*−*13*). An interesting example is neopullulanase which catalyzes the hydrolysis of α-(1,4) or α-(1,6) glucosidic linkages, as well as the transglycosylation, to form α-(1,4) or α-(1,6) linkages (*14*). Site-directed mutagenesis studies suggest that one active center is participating in the four reactions catalyzed by neopullulanase (*14*). Furthermore, Kuriki et al. have shown that the conserved aspartate and glutamate residues are involved in the active sites of neopullulanase (*14*), BE (*11*) as seen in α-amylase (*15*), and cyclodextrin glucanotransferases (*16*). Although BE, α-amylase, and SS catalyze different reactions, they can work on the same substrate. We were interested to find out whether the acidic amino acids are also involved in SS catalysis. This study indeed provided the first evidence that the conserved aspartate and glutamate residues are important for SS activity.

The importance of the carboxyl amino acid residues was demonstrated by chemical modification of SS protein with EDAC. ADPGlc completely protected SSIIb-2 from inactivation by EDAC. In contrast, amylopectin or glycogen did not protect the enzyme from inactivation, while maltodextrins partially protected the enzyme from inactivation by EDAC. These results strongly suggest a more direct role of acidic amino acids in ADPGlc binding. The difference in protection by amylopectin, glycogen, and maltodextrin may be due to the hindrance in molecular sizes. On the basis of the sequence alignment of maize SS proteins and *E. coli* glycogen

Table 2: Kinetics for SSIIb-2 and Mutants[a]

	ADPGlc kinetics				primer kinetics			
	glycogen as primer		amylopectin as primer		glycogen as primer		amylopectin as primer	
	V_{max}	K_m	V_{max}	K_m	V_{max}	apparent K_m	V_{max}	apparent K_m
SSIIb-2	118.99 ± 5.06	0.13 ± 0.02	74.86 ± 5.46	0.16 ± 0.03	97.93 ± 2.97	0.05 ± 0.01	76.06 ± 3.55	0.16 ± 0.04
D21N	4.87 ± 0.25	1.48 ± 0.03	2.77 ± 0.26	1.58 ± 0.11	4.31 ± 0.31	0.28 ± 0.03	3.51 ± 0.69	0.51 ± 0.09
D21E	13.35 ± 1.32	0.12 ± 0.02	9.30 ± 0.79	0.13 ± 0.03	14.01 ± 0.61	0.21 ± 0.03	9.86 ± 0.49	0.23 ± 0.04
D139E	25.25 ± 1.88	0.07 ± 0.02	22.27 ± 2.97	0.09 ± 0.03	30.51 ± 1.51	0.08 ± 0.01	24.85 ± 1.99	0.07 ± 0.02
E391D	17.16 ± 1.89	1.18 ± 0.14	15.05 ± 1.32	1.37 ± 0.14	15.05 ± 1.32	0.63 ± 0.06	7.44 ± 1.03	0.68 ± 0.07

[a] V_{max} values are expressed as μmol Glc/min/mg. For ADPGlc kinetics, K_m values are expressed as mM ADPGlc. Glycogen concentration was 20 mg/mL; amylopectin concentration was 5 mg/mL. For primer kinetics, K_m values are expressed as mg/mL primer. For SSIIb-2, D21E, and D139E, 1 mM ADPGlc was used; 5 mM ADPGlc was used for D21N and E391D.

synthase, we have made mutants at each of the 12 conserved acidic amino acids. Using site-directed mutagenesis and activity screening, three sites (D21, D139, and E391) were found to be important for SS activity (Table 1, Figure 6). D21 is located in the conserved domain 1 of SS which includes the conserved KTGGL sequence (Figure 6). On the basis of chemical modification and site-directed mutagenesis, the lysine residue in domain 1 has been suggested to be a putative ADPGlc binding site in *E. coli* glycogen synthase (*8*, *9*). In this study, we have found that the negative charge of the aspartate plays a role in ADPGlc binding. Evidence of the importance of the negative charge can be seen in the dramatic 10-fold increase in K_m for ADPGlc for D21N, while D21E, which maintains the negative charge, shows no change in the affinity for ADPGlc. The loss of the negative charge in D21N also shows a difference in the pH profile when compared to SSIIb-2 and D21E (Figure 4). The loss of the ionizable group in D21N and the corresponding shift in the pH profile to higher pH suggest the importance of the carboxyl group in SS catalysis. These results suggest that the conserved domain 1 (KTGGLGD) may be part of or located close to the ADPGlc binding site or catalytic center. While the ϵ-amino group of the conserved lysine residue is interacting with the anionic pyrophosphate moiety of ADPGlc, the carboxyl group of D21 may be interacting with the adenosine ring of ADPGlc. Therefore, both K15 and D21 are important for ADPGlc binding and catalysis.

When D139 in the conserved domain 2 (Figure 6) was changed to asparagine, no SS activity was detected. A conservative change at site 139 from aspartate to glutamate decreased its V_{max} to 30% of wild-type enzyme activity but showed no changes in K_m for either substrate. CD analysis did not reveal significant formation changes in this mutant. These results suggest that D139 may be an important catalytic residue. We also noted that tryptophan140 and histidine141 in domain 2 are conserved among different SS proteins (Figure 6). We are interested in studying the functions of those two amino acids to understand the importance of domain 2 in SS activity.

Mutant analysis of site E391 in domain 3 suggests that that E391 is also important for the catalytic properties of SS (Figure 6). When E391 was changed to glutamine, no SS activity was detected. A conservative change from glutamate to aspartate showed an 80% decrease in activity and an increase in apparent K_m values for substrates. It shows both the negative charge and molecular size are important for SS activity. While the K_m for ADPGlc increased 9-fold in E391D, the apparent K_m for glycogen increased 13-fold, and the apparent K_m for amylopectin increased 4-fold. Two carboxyl amino acid residues (D21 and E391) have been found to be important for ADPGlc binding. Because the CD spectra showed no major difference between the mutants and wild-type enzyme, the kinetic changes are most likely not due to a major conformational change in the protein.

It has been shown that some glucosyltransferases (*23*) require divalent cations (Mg^{2+} or Mn^{2+}) for activity and that carboxylates interact with the nucleotide sugar through a complex with a divalent cation. We found no evidence that a divalent cation or such an interaction is required for SS catalysis. When the enzyme was assayed in the presence of 0, 5, 10, or 100 mM $MgCl_2$ and $MnCl_2$, respectively, no effect on the activity was observed (data not shown). We speculate that the catalytic mechanism of SS is somewhat similar to what has been shown in amylase and cyclodextrin glucanotransferase, which involves acidic residues in the active sites (*16*, *22*). While the conserved lysine residues (*8*, *9*) and/or arginine (*10*) could interact with the anionic substrate ADPGlc, D139 and E391 may act as a general acid/base catalyst. A carbonium ion intermediate may be formed between the nonreducing end of a glucan primer and the carboxyl group of a catalytic residue (possibly D139). With the substrate intermediate being stabilized by another catalytic residue (possibly E391), the transglycosylation occurs by nucleophilic attack of ADPGlc. Indirect support for this mechanism is the finding that citrate can stimulate SS activity and increase primer affinity but has no effect on ADPGlc affinity (*6*, *7*). The effect of citrate on SS activity and glucan primer affinity could be due to the stabilization of the primer−enzyme intermediate by the carboxyl group of citrate. Although D21 is not the catalytic residue or substrate binding site, it may play a stabilizing role in ADPGlc binding and catalysis because the negative charge at D21 is important for ADPGlc binding. No activities were detected in the double mutants of D21N and D139E or D139E and E391D (data not shown). These results further indicate that D21, E139, and E391 are important for SS catalysis. To fully understand the catalytic mechanism of starch synthase, we are undertaking studies on the functions of the conserved lysine residues in ADPGlc binding and catalysis. Double mutants of lysine residues and D21, E139, or E391 are being generated and studied.

ACKNOWLEDGMENT

The authors thank Dr. Jennifer Imparl-Radosevich of ExSeed Genetics L.L.C. for her critically reading the manuscript. We also thank Chuxiong Liao at the Iowa State

University Protein Facility for performing the CD measurement.

REFERENCES

1. Keeling, P. L., Banisadr, R., Barone, L., Wasserman, B. P., and Singletary, G. W. (1994) *Aust. J. Plant Physiol. 21*, 807−827.
2. Gao, M., Wanat, J., Stinard, P. S., James, M. G., and Myers, A. M. (1998) *Plant Cell 10*, 399−412.
3. Shure, M., Wessler, S., and Fedoroff, N. (1983) *Cell 35*, 225−233.
4. Knight, M. E., Harn, C., Lilley, C. E. R., Guan, H. P., Singletary, G. W., Mu-Forster, C., Wasserman, B. P., and Keeling, P. L. (1998) *Plant J. 14*, 613−622.
5. Harn, C., Knight, M., Ramakrishnan, A., Guan, H. P., Keeling, P. L., and Wasserman, B. P. (1998) *Plant Mol. Biol. 37*, 639−649.
6. Imparl-Radosevich, J. M., Li, P., Zhang, L., McKean, A. L., Keeling, P. L., and Guan, H. P. (1998) *Arch. Biochem. Biophys. 353*, 64−72.
7. Imparl-Radosevich, J. M., Nichols, D. J., Li, P., McKean, A. L., Keeling, P. L., and Guan, H. P. (1999) *Arch. Biochem. Biophys. 362*, 131−138.
8. Furukawa, K., Tagaya, M., Inouye, M., Preiss, J., and Fukui, T. (1990) *J. Biol. Chem. 265*, 2086−2090.
9. Furukawa, K., Tagaya, M., Tanizawa, K., and Fukui, T. (1993) *J. Biol. Chem. 268*, 23837−23842.
10. Imparl-Radosevich, J. M., Keeling, P. L., and Guan, H. P. (1999) *FEBS Lett. 457*, 357−362.
11. Kuriki, T., Guan, H. P., Sivak, M., and Preiss, J. (1996) *J. Protein Chem. 15*, 305−313.
12. Buisson, G., DuJe, E., Haser, R., and Payan, F. (1987) *EMBO J. 6*, 3909−3916.
13. Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Swift, H., Thim, L., and Woldike, H. F. (1990) *Biochemistry 29*, 6244−6249.
14. Kuriki, T., Takata, H., Okada, S., and Imanaka, T. (1991) *J. Bacteriol. 173*, 6147−6152.
15. Matsuura, Y., Kusunoki, M., Harada, W., and Kakudo, M. (1984) *J. Biochem. 95*, 697−702.
16. Klein, C., and Schultz, G. E. (1991) *J. Mol. Biol. 217*, 737−750.
17. Espada, J. (1962) *J. Biol. Chem. 252*, 2891−2899.
18. Plant, A. R., Clemens, R. M., Morgan, H. W., and Daniel, R. M. (1987) *Biochem. J. 246*, 537−541.
19. Hawker, J. S., Ozbun, J. L., Ozaki, H., Greenberg, E., and Preiss, J. (1974) *Arch. Biochem. Biophys. 160*, 530−531.
20. Kloesgen, R. B., Gierl, A., Schwartz-Sommer, Z. S., and Saedler, H. (1986) *Mol. Gen. Genet. 203*, 237−244.
21. Kumar, A., Larsen, C. E., and Preiss, J. (1986) *J. Biol. Chem. 261*, 16256−16259.
22. Svensson, B. (1994) *Plant Mol. Biol. 25*, 141−157.
23. Pitcher, J., Smythe, C., and Cohen, P. (1988) *Eur. J. Biochem. 176*, 391−395.