Identification of Multiple Phosphorylation Sites on Maize Endosperm Starch Branching Enzyme IIb, a Key Enzyme in Amylopectin Biosynthesis

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Background: Starch is the major component of cereal yield, yet the biochemical regulation of its synthesis is poorly understood.

Results: Starch branching enzyme IIb is phosphorylated at three sites by two Ca²⁺-dependent protein kinases.

Conclusion: Two phosphorylation sites represent a general mechanism of control in plants, the third is cereal specific.

Significance: Identification of post-translational regulatory mechanism offers possibilities for targeted manipulation of starch.

Starch branching enzyme IIb (SBEIIb) plays a crucial role in amylopectin biosynthesis in maize endosperm by defining the structural and functional properties of storage starch and is regulated by protein phosphorylation. Native and recombinant maize SBEIIb were used as substrates for amyloplast protein kinases to identify phosphorylation sites on the protein. A multidisciplinary approach involving bioinformatics, site-directed mutagenesis, and mass spectrometry identified three phosphorylation sites at Ser residues: Ser649, Ser286, and Ser297. Two Ca²⁺-dependent protein kinase activities were partially purified from amyloplasts, termed K1, responsible for Ser649 and Ser286 phosphorylation, and K2, responsible for Ser649 and Ser297 phosphorylation. The Ser286 and Ser297 phosphorylation sites are conserved in all plant branching enzymes and are located at opposite openings of the 8-stranded parallel β-barrel of the active site, which is involved with substrate binding and catalysis. Molecular dynamics simulation analysis indicates that phospho-Ser297 forms a stable salt bridge with Arg665, part of a conserved Cys-containing domain in plant branching enzymes. Ser649 conservation appears confined to the enzyme in cereals and is not universal, and is presumably associated with functions specific to seed storage. The implications of SBEIIb phosphorylation are considered in terms of the role of the enzyme and the importance of starch biosynthesis for yield and biotechnological application.

Starch is a water-insoluble polyglucan, providing higher plants and green algae with an osmotically inert carbon store that is adapted for both short term storage (e.g. over a diurnal cycle in leaves), or longer term for the next generation (e.g. storage starch of seed endosperms). From a nutritional perspective, starch is the major caloric component of human and many livestock diets, and its varied physical properties are exploited for numerous industrial purposes. In common with other polyglucans in nature, starch is composed of linear α-(1→4)-O-linked glucans and α-(1→6)-O-linked branches; the organized positioning and frequency of branch points is a distinguishing feature of starchy, and contributes to their water insolubility (1, 2). α-(1→4)-O-Linked glucan chains are formed by NDP-glucose-dependent transferases; in plants these are multiple isoforms of ADP-glucose-dependent starch synthases (SS) (1, 4–8) (ADP-glucose: 1→4-α-D-glucan 4→α-D-glucosyltransferase; E.C. 2.4.1.21), one of which is physically associated with the starch granule (granule-bound starch synthase I), and responsible for formation of an essentially unbranched, minor component of starch called amylose. Four soluble stromal SS isoforms (SS I–IV) are responsible for the synthesis of amylopectin clusters, the major structure-defining component of the starch granule. Different SS isoforms have distinct biochemical properties and produce α-glucan chains of different length, or degree of polymerization; which are also substrates for starch branching enzymes (SBE) (1, 4–8). The latter generate α-(1→6)-branch linkages through the hydrolytic cleavage of internal α-(1→4) bonds and transfer of the released reducing ends to C6 hydroxyl groups (3). In addition to multiple isoforms of SS and SBE, starch granule biosynthesis requires debranching enzymes (E.C. 3.2.1.41 and E.C. 3.2.1.68) whose role is thought to be removal of certain branch points, which facilitates formation of water-insoluble semi-crystalline structures (4, 5).

Two classes of SBE, SBEI and SBEII, show distinct preferences for the length of preformed linear α-glucan chains hydrolyzed, and show differences in the length of glucan chain trans-
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ferred, either onto a neighboring acceptor chain (inter-chain branching), or onto the same chain (intra-chain branching). In vitro, SBEI has a high affinity for amylose, whereas the SBEII class has a higher affinity for amylpectin and transfers shorter glucan chains (degree of polymerization 6–14); the latter chains being found in amylpectin clusters (6). Many studies of plant SBE mutants support these roles; in particular, SBEII plays an important role in amylpectin biosynthesis and in defining structural organization (7–10). In cereals, the SBEII class comprises two closely related but distinct gene products with minor differences in biochemical characteristics, termed SBEIIa and SBEIIb (11, 12). The predominance of either form of SBEII is a function of the tissue and species in which they are expressed (13–15). In maize (Zea mays L.) endosperm SBEIIb is the predominant form, being expressed at ~50 times the level of the IIa form (16, 17) and is the most abundant protein in the maize amyloplast stroma (18).

An emerging aspect of our understanding of the regulation of storage starch biosynthesis is the physical association of different classes of amylpectin-synthesizing enzymes in protein complexes (19–21) within starch-synthesizing plastids, and the important regulatory role played by protein phosphorylation. The SBEII class forms a functional trimeric protein complex with SSI and SSIIa, and this complex has been implicated in amylpectin cluster biosynthesis (20, 22). The components of the trimeric protein complex eventually become entrapped within the starch granule through the glucan-binding capacity of SSIIa (22). In addition, SBEIIb has also been detected in a protein complex with SBEI and starch phosphorylase (E.C. 2.4.1.1) (19). Assembly and disassembly of a number of heteromeric protein complexes involved in amylpectin biosynthesis is regulated by protein phosphorylation (19, 20, 23). In the trimeric protein complex found in cereal endosperm amyloplasts SBEIIb is phosphorylated. In addition un-complexed SBEIIb is also found phosphorylated in the plastid stroma (19, 23, 24). The catalytic activity of SBEII forms in monocot plastids has also been shown to be modulated by the phosphorylation state of the enzyme (19). Regulation of the amylpectin synthesis pathway (and of heteromeric protein complexes) by protein phosphorylation is axiomatic of a carefully controlled metabolic process, ultimately impacting the carbon budget of the plant.

A prerequisite to understanding the complex signal transduction system regulating amylpectin biosynthesis in plants is the identification of phosphorylation sites on defined phosphoproteins to determine the effects of phosphorylation on target proteins and protein complexes, and identify protein kinases and protein phosphatases involved. To date, none of the regulatory proteins involved in starch metabolism have been characterized in this manner. This article reports on the identification of multiple Ser phosphorylation sites on SBEIIb, a key enzyme in amylpectin biosynthesis, and component of a number of heteromeric protein complexes whose activity influences the functional properties of starch. Two distinct Ca^{2+}-dependent protein kinase activities have been partially purified that show differential specificity for Ser residues on SBEIIb.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Isolation of Amyloplasts from Developing Endosperm—Maize plants (a common maize inbred background termed CG102) were grown in a field in Guelph, Ontario, and tagged at pollination. Whole cobs were harvested 20–25 days after pollination and used to prepare amyloplasts within 4 h of harvest.** Maize endosperm amyloplasts were isolated using a modification of the methods described by Tettlow et al. (25). Plastids were osmotically lysed in a buffer containing 100 mM Tricine/KOH, pH 7.8, 1 mM Na_{2}EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl_{2}, and a protease inhibitor mixture (ProteCEASE (G-Biosciences) used at 10 μl/cm^{3}) and stored at ~80 °C until future use. Stromal proteins were separated from membranes and other particulate material by high speed centrifugation according to previously described methods (25). In general 5 ml of amyloplast suspension (1–2 mg of protein/ml) was yielded from 1 maize cob.

**Cloning, Expression, and Purification of Recombinant SBEIIb and Its Truncation Products—The plasmid vector (pET 29, Novagen catalog number 69871-3) containing SBEIIb (amino acids 62–799 referring to its full-length cDNA sequence, GenBank™ accession number L08065) with fused N-terminal S-tag was kindly provided by Dr. Alan Myers (Iowa State University).** Various segments of the coding sequence of the SBEIIb cDNA were PCR amplified from the pET 29 vector. PCR were set up to produce fragments of different lengths, and products were used to create truncated versions of recombinant SBEIIb. Individual forward primers were designed to initiate amplification at bp 385, 642, and 880 of the SBEIIb cDNA sequence. The sequences of the respective primers were: 5'-TTCCATGGCT-CAAGGCTATAAGTACC-3', 5'-TTCCATGGCTCTGCTT-AACAAATGC-3', 5'-GGCCATGGTGCGGAGTAGTACG-3'. A sequence 5'-AGGTCCGAGTCCTCCACCTGAGCATAG-3' at 2400 bp was used as a reverse primer for all three fragments resulting in N-terminal truncated versions of SBEIIb: 128–799 aa (ΔN1), 214–799 aa (ΔN2), and 291–799 aa (ΔN3) (Fig. 1). Three reverse primers were designed to amplify PCR products at bp 385, 642, and 880 of the SBEIIb cDNA sequence. The sequences of the respective primers were: 5'-TACGTCGACTCAGGCTTGTGCAATGG-3', 5'-GCGTCGACTCATGGCGGACCTTTGT-3', 5'-CGTGCGACTACCTTCTAAACACACCT-3'. The same forward primer, 5'-CGATCTCTCTCATGGTTGCAATGG-3', at 184 bp was used for all three fragments resulting in C-terminal truncated versions of SBEIIb: 62–732 aa (ΔC1), 62–646 aa (ΔC2), and 62–562 aa (ΔC3) (Fig. 1). All forward primers create a Ncol restriction site (underlined), whereas all reverse primers create a SalII restriction site (underlined). All reverse primers create stop codon (bold). PCR was performed in a 50-μl solution that contained 50 ng of plasmid, 125 ng each of forward and reverse primers, 1 μl of Pfu Turbo polymerase (Stratagene catalog number 600250), 5 μl of 10× reaction buffer, and 1 μl of a 25 mm stock solution of all four deoxyribonucleoside 5’-triphosphates (Invitrogen catalog number 18427013). The PCR conditions were: 94 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR products were digested with Ncol and SalII restriction enzymes,
purified on a 1% (w/v) agarose gel and extracted using a Gel Extraction Kit (Qiagen catalog number 28704). These fragments were ligated into the pET29 vector that had been digested with NcoI and SalI. The resulting pET29 vector constructs with various inserts were transformed and amplified in *Escherichia coli* XL1-Blue competent cells (Stratagene catalog number 200249) and sequenced to confirm synthesis of the correct constructs. Recombinant plasmids containing full-length and truncated versions of SBEIIb were transformed into ArcticExpress competent cells (Stratagene catalog number 230193) and proteins were expressed by inducing with 1 mM isopropyl β-D-thiogalactopyranoside at 10 °C, 250 rpm for 24 h. 

*E. coli* (ArcticExpress) cells were collected by centrifugation and lysed using “BugBuster Protein Extraction Reagent” (Novagen catalog number 70584-4). Recombinant proteins were purified from inclusion bodies using a Protein Refolding Kit (Novagen catalog number 70423) according to the manufacturer's instructions. Recombinant proteins were further purified by size-exclusion chromatography according to previous protocols (22) to separate catalytically active monomers from inactive aggregates. Monomeric (non-aggregated) forms were used in all experiments. Recombinant proteins were stored either at −20 °C for 1 month or at −80 °C in 40% (v/v) glycerol for 2–3 months and catalytic activity was monitored by zymogram (in gel assay) before experimental work according to Liu et al. (23).

**Site-directed Mutagenesis**—The QuikChange site-directed mutagenesis kit (Stratagene, catalog number 200518) was used to make point mutations in the recombinant SBEIIb. Serine residues were replaced by alanine in the multiple sites: Ser147, Ser204, Ser286, Ser297, Ser298, Ser568, Ser598, Ser649, Ser659, Ser699, and Ser705. Double and triple mutants were created by replacing Ser residues by Ala in the SBEIIb recombinant mutant protein. The QuikChange site-directed mutagenesis was performed using PfuTurbo™ DNA polymerase II 2.5 units/50 µl of reaction mixture, which also contained 5 µl of 10× buffer from the kit, 50 ng of vector, 125 ng of each forward and reverse primers, 1 µl of 50 mM MgSO₄, and 1 µl of 10 mM dNTP mixture (Invitrogen). Cycling parameters were: 18 cycles, 95 °C for 30 s, 55 °C for 1 min, 68 °C for 12 min followed by the final extension at 72 °C for 10 min. PfuTurbo DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. A supercoiled double-stranded DNA vector pET 29 with an insert of SBEIIb and a pair of oligonucleotide primers for each mutant containing the desired mutation were utilized. The coding sequence for Ser was substituted for coding sequence for Ala in each set of primers. The primer sequences used for site-directed mutation of SBEIIb were as follows: forward, 5'-CTCTATAGAAGAATCGCTGGAAGACATTTGAGAC-3'; reverse, 5'-GGTGATCATTAGAGCCGAATATGAC-3'; for Ser147; forward, 5'-CCACCGAGATCTGATGAGGAGATGAGG-3'; reverse, 5'-GTCATATTGACCGACTTAACCAAC-3'; for Ser204; forward, 5'-CTTCAACGCACCGGAATACGAGAAACCA-3'; reverse, 5'-GTCATATTGACCGACTTAACCAAC-3'; for Ser286; forward, 5'-GGATGCTAGGCTGCCGGAAACCAGATATAACAC-3'; reverse, 5'-GTCATATTGACCGACTTAACCAAC-3'; for Ser297; forward, 5'-GGAAATGAGTGCACCACCCCGAA-CCGAAGATAAACAAC-3'; reverse, 5'-GTGGTTATCTTCCGTTCCCGGAGAATATCCACC-3'; for Ser298; forward, 5'-GGTAACTATGGAACCTCATGACAACC-3'; reverse, 5'-GCTTGATCATTAGAGCTTCAGCTATAAGTATTACAC-3'; for Ser568; forward, 5'-CCCTGAGAAGCTGCAAACATCTTACCATTG-3'; reverse, 5'-CAAATGACATGACGAGGGGAG-3'; for Ser598; forward, 5'-CGCAAAGACCTTCCAAGTTTACGCTACC-3'; reverse, 5'-GCCAATACTCTACGCTTACGTTTTGTTAATTTCCCTG-3'; for Ser649; forward, 5'-GAATCATGACGAGCTTCATGCA-3'; reverse, 5'-GGAAATAACCTACGCTTACGTTTTGCG-3'; for Ser659; forward, 5'-CCAGATATTGGCCGGAAACATGAGGAGG-3'; reverse, 5'-GCCAATACTCTACGCTTACGTTTTGTTAATTTCCCTG-3'; for Ser699; and forward, 5'-CCACGATATTTGGCCGGAAACATGAGGAGG-3'; reverse, 5'-GTCATATTGACCGACTTAACCAAC-3'; for Ser705 (mutated nucleotides are in bold and codons for Ala are underlined). Incorporation of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. Following temperature cycling, the product was treated with 1 µl of DpnI (10 units/µl) per 10 µl of PCR product at 37 °C for 2 h. The DpnI endonuclease (target sequence: 5'-GmA6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and select for mutation-containing synthesized DNA.

DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to DpnI digestion. 4 µl of nicked vector DNA incorporating the desired mutations was then transformed into 50 µl of Epicurian Coli® XL1-Blue competent cells. 200 µl of each transformation reaction was plated on LB-ampicillin agar with 20 µl of 10% (w/v) X-Gal and 20 µl of 100 mM isopropyl β-D-thiogalactopyranoside. Plates were incubated at 37 °C for 16–18 h. White colonies were screened for mutated sequences, and isolated DNA samples from white colonies were sequenced to confirm the mutation sites. Samples of the pET 29 vector containing inserts of SBEIIb mutants were transformed into Arctic Express cells and expression and purification of mutant recombinant proteins was carried out as for expression and purification of recombinant SBEIIb described above.

**Enzyme and Protein Assays and Kinetic Studies**—SBE activity was assayed indirectly by stimulation of incorporation of 14C from [U-14C]-α-D-glucose 1-phosphate into glucan by glycogen phosphorylase α, according to methods previously described (20) and also by native-PAGE zymograms according to the methods reported previously (23). SBEIIb kinase activity was determined by direct phosphorylation of recombinant maize SBEIIb (see below) and using a synthetic peptide kinase assay. In *vitro* peptide kinase assays were performed as previously described (26) with reaction conditions for SBEIIb kinase optimized for all peptides; all reactions were linear with respect to time and protein content (data not shown). The assay mixture (25 µl) contained 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 200 µM [γ-32P]ATP, 5 mM MgCl₂, 200 µM synthetic peptide, and 10 µl of amyloplast lysate or partially purified kinase was incubated at 25 °C for 10 min. Following incubation the reaction was terminated by removing 15 µl of the reaction mixture and pipetting

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onto 2-cm² phosphocellulose P-81 paper (Whatman) and allowing the sample to soak into the paper. The phosphocellulose paper was washed 3 times (5 min/wash) by swirling in 0.5 liters of 1% (v/v) orthophosphoric acid, followed by a final wash in 0.5 liters of acetone. P-81 paper was dried on paper towels and placed in scintillation vials with 4 cm³ Ecoscint (Dial Med) and radioactivity was quantified using a Beckman Coulter LS6500 Multi-purpose liquid scintillation counter. Protein concentrations were determined using the Coomassie Blue G-250 dye binding method using bovine γ-globulin (Sigma) as the protein standard (27).

Synthetic Peptides—Synthetic peptides based on amino acid sequences of SBEIIb were purchased from CanPeptide Inc. (Pointe-Claire, Québec, Canada) and used as substrates for in vitro protein kinase assays (peptide sequences are summarized in Table 1). Each peptide contained an additional three Arg residues at the C terminus to bind to the phosphocellulose paper.

In Vitro Phosphorylation of Recombinant SBEIIb and Amyloplast Stromal Proteins—In vitro phosphorylation of recombinant proteins was performed by two methods: either phosphorylating recombinant proteins followed by attaching them to S-protein-agarose beads (Novagen) or by attaching recombinant proteins to S-agarose beads first prior to phosphorylating them; in both cases using amyloplast lysates as a source of protein kinase. For the first method 20 μg of monomeric SBEIIb was incubated for 30 min with 100 μl of amyloplast lysate (1–1.5 mg of protein/ml) or partially purified protein kinase in the presence of 0.1 mM [γ-32P]ATP (0.5 μCi, PerkinElmer Life Sciences), 10 μl/ml of protease inhibitors (Plant Protease-Arrest), and 10 μl/ml of protein phosphatase inhibitors (PhosphataseArrest, G-Biosciences) in a total volume of 135 μl, then 100 μl of 50% (w/v) S-agarose slurry was added to each sample and the mixture incubated for 1 h to bind recombinant SBEIIb to the beads. In the second method 20 μg of monomeric recombinant SBEIIb was incubated with 100 μl of 50% (w/v) S-protein-agarose slurry at room temperature on a rotator for 1 h in the presence of protease inhibitors (10 μl/ml). Unbound proteins were removed by washing with 10 ml of buffer A containing, 20 mM Tris-Cl, pH 7.8, 0.15 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM DTT. The washed beads containing recombinant SBEIIb were then incubated with a source of protein kinase and [γ-32P]ATP as for the phosphorylation reactions described above at room temperature with gentle agitation for 30 min. In both experiments reactions were stopped by adding Na2-EDTA to a final concentration of 20 mM and unbound proteins and non-incorporated ATP were washed out from samples with 10 ml of wash buffer B containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20, 1 mM DTT. 50 μl of SDS loading buffer was added to each sample and samples were heated at 95 °C for 5 min prior to SDS-PAGE. Amyloplast stromal proteins (100 μl, 1–1.5 mg of protein/ml) were phosphorylated in vitro by incubation with 0.1 mM [γ-32P]ATP (0.5 μCi) in a total volume of 135 μl. Reactions were incubated at room temperature with gentle agitation for 30–45 min and terminated by adding Na2-EDTA to a final concentration of 20 mM.

Phosphoamino Acid Analysis—Phosphorylated SBEIIb was separated by SDS-PAGE and extracted from gels (5–10 gel slices per sample), and partial acid hydrolysis of the sample was performed in 5.7 n HCl for 1 h at 110 °C. The released phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis and autoradiography using methods described previously (28).

In-gel Trypsin Digestion—In-gel trypsin digestion of 32P-labeled SBEIIb was conducted according to the methods described previously (19). Silver-stained gel pieces were first washed with 30 μl of 15 mM potassium ferricyanide, 50 mM sodium thiosulfate followed by successive rinses with deionized water, 50 mM ammonium hydrogen carbonate (NH4HCO3) buffer, and acetonitrile. This destaining step was omitted in the processing of Coomassie Blue-stained bands. Cys residues were reduced with 10 mM DTT and derivatized by treatment with 100 mM iodoacetamide. After further washing with NH4HCO3 buffer, the gel pieces were dehydrated with acetonitrile and dried at 60 °C before addition of modified trypsin (10 μl of a solution containing 6.5 ng of protein/μl in 25 mM NH4HCO3; Promega, Madison, WI). Digestion proceeded for 1 h at room temperature (to rehydrate the gel slice in the trypsin solution) followed by incubation at 37 °C for 16–18 h, and products were recovered by sequential extractions with 25 mM NH4HCO3, 5% (v/v) formic acid, then acetonitrile. The pooled extracts were lyophilized and dissolved in 0.1% (v/v) formic acid either for MS analysis or for purification of phosphopeptides by reversed-phase high performance liquid chromatography (RP-HPLC, see below).

Purification of Phosphopeptides by RP-HPLC—Tryptic digest products of phosphorylated SBEIIb were separated by RP-HPLC using a Gemini-NX 3 μ C18 110A column on an Agilent 1100 Series HPLC pump according to the manufacturer’s instructions. The mobile phase was a gradient of HPLC grade water with 0.1% (v/v) formic acid and acetonitrile with formic acid, which was converted from 95 to 0% water over 38 min. Absorbance at 217 nm was measured to determine peptide elution. Fractions containing 32P-labeled phosphorylated peptides were determined by a Geiger counter and liquid scintillation counting and subsequently analyzed by mass spectrometry (MS).

MS Analyses—For MS-based analysis of SBEIIb phosphorylation sites the protein was separated by SDS-PAGE and SBEIIb was subjected to in-gel digestion prior to MS analysis. Coomassie Blue stain was removed from the isolated bands with water containing 50% (v/v) acetonitrile. The samples were washed twice with 100 mM NH4HCO3 and reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 for 30 min at 60 °C and then cooled to 21 °C. Cysteine alkylation was performed with 20 mM iodoacetamide in 100 mM NH4HCO3 in the dark for 1 h. Excess iodoacetamide was removed with two washes of 100 mM ammonium bicarbonate. The proteins were digested at 37 °C using 0.4 μg of endoproteinase Glu-C (Endo-Glu-C, Sigma) per gel band for 16 h, then with 0.4 μg of endoproteinase Lys-C (Endo-Lys-C, Sigma) per sample for 16 h. Gel bands were washed with 100-μl volumes of water, then 50% (v/v) acetonitrile, and washes were pooled, dried in a centrifugal concentrator, dissolved in aqueous 0.1% (v/v) formic acid, and purified using C18 Zip Tips (EMD Millipore, Darmstadt, Germany). Purified Endo-Glu-C/Lys-C digests were analyzed by online
nanoflow HPLC-MS/MS using a NanoLC-Ultra 2D HPLC pump coupled to a Nanoflex CHiPLC system and a TripleTOF 5600 mass spectrometer (AB Sciex, Concord ON, Canada). Peptides were loaded and washed on a 0.5-mm long, 200-μm diameter trap (AB Sciex), and resolved on a 150-mm long, 75-μm diameter chromatographic column (AB Sciex). The trap and the analytical column packing material consisted of 3-μm diameter C18 particles with 120-Å pores. Peptide separation was performed using a binary mobile phase gradient. Mobile phases A and B contained 0.1% (v/v) formic acid in water and acetonitrile, respectively. The following mobile phase B compositions were used: 2% at 0 min, 35% at 45 min, 80% from 46 to 50 min, 2% from 51 to 60 min. The mass spectrometer was operated in positive ion mode with an electrospray voltage of 2300 V. The MS data acquisition cycle consisted of a single MS scan with a range of 400 to 1250 Da followed by MS/MS scans from 100 to 1800 Da of the most abundant ions detected in the preceding MS scan. The number of MS/MS scans per MS scan was limited to 20. Peptides and phosphopeptides were identified from the tandem mass spectra using ProteinPilot software with phosphorylation emphasis and amino acid substitutions specified in the search parameters. Separate searches of the dataset were conducted using the RefSeq Z. mays library, which contained 22,444 sequences representing 7,089,707 residues for peptide identifications (downloaded November 14, 2012).

Partial Purification of SBEIIb Kinase—SBEIIb kinase was partially purified from amyloplasts via various column chromatography steps at 4 °C using an AKTA Explorer FPLC (Amersham Biosciences). Between 5 and 10 mg of amyloplast stromal proteins (10–12 ml of plastid lysate) were loaded onto a 1-ml HiTrapDEAE FF column (GE Healthcare) at a flow rate of 0.5 ml/min. The column was washed with running buffer containing 100 mM Tricine/NaOH, pH 7.5, 7.5 mM MgCl2, 1 mM DTT until the A280 had returned to baseline and SBEIIb kinase activity was eluted using a 25-mL linear gradient from 0 to 1 M KCl in running buffer. Catalytically active fractions were determined using both the peptide kinase assay and phosphorylation of recombinant SBEIIb. Active fractions were pooled, desalted on a PD-10 column (Amersham Biosciences), and loaded onto a 1-ml HiTrap Blue HP column (GE Healthcare). The column was washed with running buffer until the A280 returned to baseline, then SBEIIb kinase activity was eluted using a 20-mL gradient from 0 to 1.5 M KCl in running buffer.

Electrophoresis and Immunoblotting—Protein samples were separated on 10% SDS-PAGE and Western blot analyses were performed using methods described previously (19). Proteins were identified by cross-reaction with either anti-SBEIIb or anti-S-tag antibodies (Abcam catalog number ab24838) in dilutions of 1:5000 or 1:3000, respectively. Peptide-specific polyclonal rabbit antisera targeted to maize SBEIIb was prepared and purified according to methods described by Liu et al. (23).

Homology Modeling of SBEIIb—The structural and sequence homologs of maize SBEIIb were identified using TM-align program (29) and Blastp (30), respectively. In this analysis, only the mature protein sequence (coding for 738 amino acids) was used and the 61-amino acid N-terminal transit peptide region was not considered. Sequence alignment was performed using MAFFT, Tcoffee-EBI, and ClustalW. Maize SBEIIb structure prediction was carried out using six tools: CPHmodels3,4 (31), ESyPred3D5 (32), SWISS-MODEL Workspace6 (33), Robetta7,8 (34), I-TASSER8,9 (35), and Phyre 2.010 (36). These tools used one or a combination of homology, ab initio, or threading/fold recognition modeling methods. Of these six, the homology structure produced by I-TASSER was chosen for subsequent MD analysis.

Molecular Dynamics Simulation of SBEIIb—Molecular dynamics simulation of the maize SBEIIb homology structure was performed using the GROMACS software package (37) version 4.5.5 and the Gromos96 ffG53a6 force-field (38). This force-field was parameterized to recognize phosphorylated Ser residues, as was previously done for the Gromos96 43a1p force-field, with partial charges and van der Waals values for the atoms in the phosphate group obtained from Hansson et al. (39). All simulations were run on the Compute Canada/SHARCNET high performance computing cluster. Eight different SBEIIb structural models were used in these experiments: (i) unmodified SBEIIb, (ii) SBEIIb phosphorylated at Ser286 (SBEIIb_phos286), (iii) SBEIIb phosphorylated at Ser297 (SBEIIb_phos297), (iv) SBEIIb phosphorylated at Ser649 (SBEIIb_phos649), (v) SBEIIb doubly phosphorylated at Ser286 and Ser297 (SBEIIb_phos286_297), (vi) SBEIIb doubly phosphorylated at Ser286 and Ser649 (SBEIIb_phos286_649), (vii) SBEIIb doubly phosphorylated at Ser297 and Ser649 (SBEIIb_phos297_649), and (viii) SBEIIb triply phosphorylated at Ser286, Ser297 and Ser649 (SBEIIb_phos286_297_649). In all cases, the appropriate Ser residues were phosphorylated using the SYBYL-X 1.3 molecular modeling suite (SYBYL, Tripos Associates, St. Louis, MO) and the charge on each phosphate group was −2. Each model was prepared in an identical way for molecular dynamics simulation. The protein was centered in a virtual cubic box, allowing at least a 1-nm gap between the protein and the edge of the box. The box was solvated with water molecules using the spc216 water model (40), and ions Na+ and Cl− ions were added such that the final salt concentration was 0.15 M and the overall net system charge was neutral. This solvated and neutralized system was energy minimized using the steepest decent minimization algorithm to a maximum overall force of <1,000 kJ mol−1 nm−2, and this was followed by two 100-ps steps in which the system was equilibrated at 298 K and 1 bar, whereas the protein position was restrained. Molecular dynamics simulation were then carried out for a total of 100 ns utilizing velocity rescaling with a stochastic term for temperature coupling (41) and Berendsen isotropic pressure coupling (42). The trajectories were visualized using VMD (43) version 1.9.1 and analysis was done using a number of GROMACS utilities.

Statistics—Data were analyzed using the Student’s t test, and deemed significant at p < 0.05.

RESULTS

32P Labeling of N- and C-terminal Truncations of Recombinant Maize SBEIIb—Previous in vitro 32P labeling studies in maize endosperm amyloplasts have shown that SBEIIb is rapidly phosphorylated by a plastidial protein kinase activity (24). Immobilized recombinant SBEIIb was also readily phosphorylated in the presence of amyloplast lysates, a source of protein

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kinase and [γ-^32P]ATP (Fig. 1B) and formed the basis for phosphorylation experiments with truncated forms of SBEIIb (see below). Phosphoamino acid analysis of both native and recombinant forms of phosphorylated SBEIIb indicated that phosphorylation occurred on one or more serine (Ser) residues (data not shown).

To locate the approximate positions of phosphorylation sites on the mature protein (i.e. the form lacking the putative transit peptide predicted to be in amyloplasts) a series of N- and C-terminal truncations of recombinant maize SBEIIb was produced (Fig. 1A) and heterologously expressed in E. coli and used as substrates for SBEIIb kinase(s) present in amyloplasts. The recombinant maize SBEIIb used in these experiments was the mature form, lacking the 61-amino acid transit peptide. The truncations were designed to give maximum coverage of the mature sequence of SBEIIb, although none of the truncated products were catalytically active (data not shown). Fig. 1B shows no loss of phosphorylation of SBEIIb with the ΔC1 truncation when compared with the full-length, mature protein, but almost complete loss of ^32P labeling in the ΔC2 and ΔC3 truncations.

Site-directed Mutagenesis of Putative Ser Residues in the C Terminus—Results from ^32P labeling of the C-terminal truncations of recombinant maize SBEIIb (Fig. 1B) suggest that the phosphorylation site(s) responsible for most of the ^32P labeling are in the C terminus, specifically from amino acid residues 646–732. A series of site-directed mutants of SBEIIb were produced in which all Ser residues within this C-terminal region were individually mutated to Ala, and the resulting recombinant proteins were treated with [γ-^32P]ATP and amyloplast lysates as a source of protein kinase (Fig. 2B). The results show that the Ser^649 > Ala mutation caused substantial loss of ^32P labeling compared with the wild-type protein, whereas other Ser > Ala mutations in the C terminus caused no loss in labeling (Fig. 2B). Loss of the Ser^649 residue and its replacement by Ala caused no measurable change in the catalytic activity of the mutant SBEIIb compared with the wild-type protein as
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32P Labeling of site-specific SBEIIb mutants. Recombinant maize SBEIIb and a series of site-directed Ser → Ala SBEIIb mutants that covered all C-terminal Ser residues from amino acids 646 to 732 were immobilized on S-agarose beads and incubated with 0.1 mM [γ-32P]ATP and amyloplast stroma (0.1 mg of protein) as a source of protein kinase for 30 min at 25 °C. A and C, immobilized proteins on beads were washed and 5 μg separated by SDS-PAGE, electroblotted to a nitrocellulose membrane, and visualized using anti-S-tag antibodies. B and D, 32P labeling of the electroblotted recombinant proteins visualized by autoradiography.

It is notable, however, that the Ser649 > Ala mutation showed some residual phosphorylation at one or more other sites. Because phosphorylation of Ser649 appears to account for much of the radiolabeling of SBEIIb in vitro, we therefore mutated additional candidate Ser residues in the Ser649 > Ala recombinant protein. Fig. 2D shows that mutation of Ser286 or Ser297, in this background, caused further reduction in 32P labeling of the recombinant proteins. Notably, mutation of the adjacent Ser residue, Ser296, did not reduce the labeling of SBEIIb further. Phosphorylation of SBEIIb was not detectable in the Ser649–Ser286–Ser297 triple mutant (Fig. 2D).

32P Labeling of Native and Recombinant SBEIIb Is Inhibited in the Presence of a Synthetic Peptide Containing Ser649—The data in Fig. 2 show that phosphorylation of Ser649 is responsible for most of the 32P labeling of SBEIIb when amyloplast lysates are incubated with the recombinant protein in the presence of [γ-32P]ATP. A 19-amino acid synthetic peptide was generated containing the amino acid sequence surrounding Ser649, which also included three C-terminal Arg residues to allow the peptide to be employed as a protein kinase substrate in in vitro assays (26) (see below). The peptide was also used as an antagonist to the protein kinase activity responsible for SBEIIb phosphorylation (Fig. 3). Phosphorylation of both recombinant maize SBEIIb and the native protein in amyloplast lysates was strongly inhibited by micromolar concentrations of the Ser649 synthetic peptide (Fig. 3). Fig. 3B shows that many amyloplast stromal proteins are phosphorylated following a 30-min incubation with 0.1 mM [γ-32P]ATP, but notably only phosphorylation of SBEIIb is inhibited by the Ser649 synthetic peptide.

Partial Purification of SBEIIb Protein Kinase Activity Shows SBEIIb Is Phosphorylated at Multiple Sites by More Than One Plastidial Protein Kinase—The protein kinase(s) responsible for phosphorylation of SBEIIb were partially purified from amyloplast stromal preparations using anion exchange (DEAE-Sepharose) and Blue-Sepharose chromatography columns. SBEIIb protein kinase activity was assayed using the Ser649 synthetic peptide (PRGPQRPSGKFIPGNRRR) and recombinant SBEIIb proteins as substrates, and eluted as a single activity peak from the DEAE column in 0.28–0.45 M KCl (data not shown). Following binding and elution from a Blue-Sepharose column, the SBEIIb kinase-active fraction resolved into two peaks of protein kinase activity designated K1 and K2 (Fig. 4). SBEIIb kinase activity in the eluted fractions was measured using 32P labeling of recombinant maize SBEIIb and the Ser649 synthetic peptide assay (Fig. 4); both assays were in agreement in terms of their detection of SBEIIb kinase activity eluting from the different chromatography columns. When using recombinant SBEIIb as a substrate for protein kinase activities eluting from the column, it can be seen that 32P-labeled bands of lower molecular mass are also observed in fractions active for SBEIIb kinase (Fig. 4). These minor bands represent truncated, expressed forms of the recombinant maize SBEIIb whose identity was confirmed by Western blotting, but are not evident from Ponceau S staining, suggesting they are minor contaminants (Figs. 2C and 3A). To examine the possibility of other phosphorylation sites on SBEIIb, in addition to Ser649, we also assayed the Blue-Sepharose column eluant fractions using the recombinant S649A SBEIIb mutant as substrate for the SBEIIb kinase(s). The elution profile of protein kinase activity eluting from the Blue-Sepharose column using the site-directed mutant lacking the Ser649 phosphorylation site was similar to that of the wild-type protein. By contrast, phosphorylation of the S649A mutant was observed in the fractions corresponding to K1, with very low phosphorylation observed in the fractions corresponding to K2 (Fig. 4B).

Identification of Putative Phosphorylation Sites on SBEIIb—Phosphorylation of the S649A mutant by the K1 protein kinase fraction (Fig. 4B) indicates that SBEIIb has more than one phosphorylation site. We employed a combination of bioinformatics-driven approach and MS/MS to identify other phos-
phosphorylation sites on SBEIIb. A number of putative SBEIIb phosphorylation sites were predicted by NetPhos2.0, and sites with high probability ($p > 0.9$) were tested as possible substrates for the SBEIIb kinase(s) using synthetic peptides with triple C-terminal Arg tails as for the Ser$^{649}$ peptide (Table 1). The maximum catalytic activity of the SBEIIb kinase was determined using the various synthetic peptides under optimal conditions previously determined for the Ser$^{649}$ synthetic peptide. Table 1 shows that measurable kinase activity was found with three peptides, Ser$^{649}$, Ser$^{286}$, and Ser$^{297}$ using amyloplast lysates as a source of protein kinase, but others as well. Importantly, the phosphorylation sites on these three peptides, phosphorylated by amyloplast lysates, were also identified in the mature protein using MS/MS (see below). Mutation of either of Ser$^{286}$ and Ser$^{297}$ residues to Ala in recombinant SBEIIb resulted in phosphorylation of the Ser$^{649}$, Ser$^{286}$, and Ser$^{297}$ peptides with significantly reduced (4-fold) catalytic activities of both K1 and K2 with the Ser$^{649}$ synthetic peptide by up to 400-fold (activities of K1 and K2 in the presence of 1 mM Na$_2$-EGTA were 0.4 and 2.6 nmol/mg of protein/h, respectively, compared with 411 and 358 nmol/mg of protein/h in the presence of 50 mM Ca$^{2+}$, respectively), and full activity was restored on addition of 5 mM or greater Ca$^{2+}$ ions. Additions of 1 mM Na$_2$-EGTA reduced kinase activities of both K1 and K2 with the Ser$^{649}$ synthetic peptide by up to 400-fold (activities of K1 and K2 in the presence of 1 mM Na$_2$-EGTA were 0.4 and 2.6 nmol/mg of protein/h, respectively, compared with 411 and 358 nmol/mg of protein/h in the presence of 50 mM Ca$^{2+}$, respectively), and full activity was restored on addition of 5 mM or greater Ca$^{2+}$ ions.

**MS/MS Analysis of Phosphorylation Sites on SBEIIb**—Phosphorylation at Ser$^{286}$ was detected in trace amounts following K2 treatment and in greater abundance in K1-treated samples by LC-MS/MS. Serine and tyrosine in the Glu-C/Lys-C peptide SRLYE are both potential phosphosites, however, product ion spectra showed serine to be the modified residue (Fig. 5A). A complete y-ion series matched that expected of the unmodified peptide, whereas all a-ions and b-ions present were phosphorylated. A peak at $m/z$ 136.1 was attributed to an immunion ion of unmodified tyrosine.

The Ser$^{297}$ phosphopeptide was observed exclusively in K2-treated SBEIIb, from which it was identified with 99% confidence by a ProteinPilot search against the whole Z. mays library. The peptide THVGMSSPEPK, which contains the Ser$^{297}$ site, was observed from Glu-C/Lys-C digests in both

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**FIGURE 3.** Phosphorylation of recombinant maize SBEIIb and native SBEIIb is reduced following incubation with a synthetic peptide containing the Ser$^{649}$ phosphorylation site. A, S-agarose-immobilized recombinant maize SBEIIb (5 μg) was incubated with 0.1 mM [%$^{32}$P]ATP, amyloplast stroma (0.1 mg of protein), and increasing concentrations of the peptide containing the Ser$^{649}$ SBEIIb phosphorylation site (PQPRLPSKFGNRRR). Immobilized recombinant protein was washed, separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and visualized using anti-S-tag antibodies (left panel) or $^{32}$P labeling visualized by autoradiography (right panel). B shows $^{32}$P labeling of amyloplast stromal proteins (same conditions as in Fig. 3A) with increasing concentrations of the Ser$^{649}$ peptide; left panel, Ponceau S-stained proteins following electroblotting and right panel, autoradiograph of the corresponding blot. Solid arrow on autoradiograph shows native SBEIIb (previously identified by immunoblotting with anti-SBEIIb antibodies) and open arrow indicates migration of the phosphorylated Ser$^{649}$ peptide.
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PEPK were compared with those of other peptides observed in the same runs to determine whether sensitivity was limited by low HPLC performance. Oxidized THVGMSPEPK and unmodified THVGMSPEPK had chromatographic qualities comparable with other analytes. Despite considerable difference in their areas, the peaks of the oxidized Ser^{297}-containing peptide and the unmodified Ser^{269}-containing peptide were 0.2 and 0.22 min full width at half-maximum, respectively, suggesting that HPLC performance did not selectively reduce either signal. As evidenced by its early elution time, THVGMSPEPK is a hydrophilic peptide and may not be effectively retained by the C_{18} trap. The grand averages of hydropathy for the Glu-C/Lys-C peptide THVGMSPEPK and the corresponding tryptic peptide IYETHVGMSPEPK were −0.945 and −0.764, respectively, indicating both were hydrophilic (44). The low signals of THVGMSPEPK suggest observation of the corresponding phosphopeptide was unfavorable, because MS sensitivity for phosphopeptides is lower than for peptides.

Phosphorylated Ser^{297} was not observed in Glu-C/Lys-C digests of SBEIIb, but was identified by LC-MS/MS of tryptic digests previously fractionated off-line by RP-HPLC. Consistent with its higher calculated hydrophobicity, IYETHVGMSPEPK eluted later than the Glu-C/Lys-C equivalent digest, with a retention time of 21 min. Phospho-IYETHVGMSPEPK had a retention time of 23 min. The monoisotopic ion of the triply charged tryptic phosphopeptide had a maximum signal of 65000 counts in the precursor ion scan. Whereas the signal may have increased due to C_{18} HPLC pre-fractionation, the sensitivity of the LC-MS system for this species was apparently much higher than for the Glu-C/Lys-C equivalent digest.

Potential kinase targets within the peptide IYETHVGMSPEPK include two Ser, one Tyr, and one Thr residue. Product ion spectra showed the phosphate to be absent from all but the Ser^{297} site (Fig. 5B). Abundant a_{3}, b_{3}, and b_{7} ions indicated that tyrosine was not phosphorylated, whereas b_{3}, a_{3}, b_{7}, and b_{9} ions suggested both Tyr and Thr were unmodified. The Ser closest to the C-terminal of the peptide was observed in an unmodified state within a y_{3} ion having a mass accuracy of 0.0037 Da and signal:noise ratios between 5.5:1 and 11:1. All product ions containing the Ser^{297} site also contained a phosphate. Two series of phosphorylated ions, from y_{9} to y_{13} and b_{11} to b_{13}, supported the assignment of the ninth residue within the peptide as the phosphorylation site.

A Ser^{269} phosphopeptide was present in both K1- and K2-treated recombinant SBEIIb samples. A missed Glu-C cleavage was consistently observed around the Ser^{297} site (Fig. 5B). The fragments y_{12}, y_{13}, and y_{14} containing both the C terminus and the phosphate, were detected with signal:noise values of 56:1, 150:1, and 29:1, respectively. Six prominent methionine-oxidized and unmodified forms with retention times between 12 and 13 min into the 60-min chromatographic run. The Glu-C/Lys-C peptides containing Ser^{297} had consistently low LC-MS signals. For instance, an untargeted MS/MS analysis of K2-treated wild-type SBEIIb, resulted in relative signal intensities of 1 and 8 and 78 for the monoisotopic precursor ions of oxidized THVGMSPEPK, phosphorylated WIDFRPGPQLPSGK (Ser^{649}), and non-phosphorylated WIDFRPGPQLPSGK, respectively. The concentrations of these species should be comparable because all originated from a single protein, however, signals of the Ser^{269} containing peptide and phosphopeptide were orders of magnitude higher than those of the Ser^{297} containing peptide, suggesting the analytical platform had differential sensitivity for these analytes. Chromatograms of the oxidized and unmodified peptide THVGMS-
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### TABLE 1
Synthetic peptides used as substrates for SBEIIb kinase

Position of the Ser (highlighted) in the mature SBEIIb sequence. All synthetic peptides contained a triple Arg tail for use in in vitro assays with 32P-labeled ATP. Prediction scores are for the Ser-containing sequence from SBEIIb based on NetPhos prediction. Activities given are for standard assay using plastid lysates as a source of protein kinase, values represent the mean of at least three separate determinations and are reproducible within ±20% (S.E.) of the mean.

| Position of serine in SBEIb | Corresponding sequence of peptides with putative phosphorylation sites (serine) | Predicted scores for putative phosphorylation sites | $V_{\text{max}}$ (nmol/mg/h) | Amyloplast peptide kinase |
|-----------------------------|---------------------------------------------------------------------------------|-----------------------------------------------|----------------------------|-------------------------|
| Ser147                      | LYRRIRS ↓ DIDEHGRRRR                                                             | 0.995                                        | 14.9                       |
| Ser206                      | PNA DRM↓ KNEFGWRRR                                                               | 0.979                                        | 4.4                        |
| Ser206                      | AOPKR KPS ↓ LIERRR                                                              | 0.995                                        | 37.3                       |
| Ser297                      | ETHV GMS ↓ APEKINRRR                                                             | 0.990                                        | 8.9                        |
| Ser297, Ser298              | ETHV GMS ↓ PEPKINRRR                                                             | 0.766                                        | 8.9                        |
| Ser298                      | ETHV GMS↓ S↓ PEPKINRRR                                                          | 0.990, 0.766                                | 5.7                        |
| Ser286, Ser297              | MALORPS ↓ TPIDDRR                                                               | 0.931                                        | 0.9                        |
| Ser286                      | PRGPOQLPS ↓ GKEIPGNRRR                                                           | 0.995                                        | 71.9                       |
| Ser286                      | KFIPGNNS ↓ YDKCRRR                                                              | 0.017                                        | 6.7                        |

### TABLE 2
Saturation kinetics of SBEIIb kinases K1 and K2

Partially purified K1 and K2 protein kinase fractions from amyleplasts were used in standard synthetic peptide kinase assays in which ATP or the synthetic peptide was varied. K252a was used in the optimized assay. Data represent the mean of at least four separate determinations and are reproducible within ±15% (S.E.) of the mean.

| Peptide   | Kinase | $K_m$ for peptide ($\mu$M) | $K_m$ for ATP ($\mu$M) | $V_{\text{max}}$ (nmol/mg/h) | $IC_{50}$ for K252a ($\mu$M) |
|------------|--------|---------------------------|------------------------|-----------------------------|-------------------------------|
| Ser649     | K1     | 10.7                       | 57.7                   | 402                         | 4.7                           |
| Ser649     | K2     | 11.0                       | 58.9                   | 352                         | 23.8                          |
| Ser286     | K1     | 27.6                       | 48.0                   | 59                          | N/A                           |

Peaks corresponded to fragments having undergone phosphoric acid loss, an energetically favored fragmentation pathway of phosphopeptides. A series of N-terminal fragments, which did not contain the Ser, were without modifications. Using the combined MS/MS evidence, the presence and location of the phosphate at Ser649 in both K1-treated and K2-treated SBEIIb were deduced.

Untreated SBEIIb, as well as protein kinase-treated SBEIIb S649A and a SBEIIb S286A/S297A/S649A triple mutant were digested then analyzed by LC-MS/MS under conditions identical to those used for kinase-treated wild-type SBEIIb to further assess the kinase dependence of the phosphorylation. Although unmodified peptides containing Ser649, Ser286, and Ser297 were observed in the untreated controls, no phosphorylation of these sites was detected in these samples (data not shown).

Modeling and Molecular Dynamics Simulation Analysis of SBEIIb—Structural homology modeling and molecular dynamics simulations (MD) were carried out on SBEIIb to determine the three-dimensional location of the phosphorylation sites at Ser286, Ser297, and Ser649 and to gain insights into the possible effects of phosphorylation on the conformation of the enzyme. SBEIIb is produced as a nuclear-encoded protein with 799 amino acids and it is this sequence that was used in homology modeling. Of all proteins with published structures, starch branching enzyme I (SBEI) from rice (Oryza sativa L.) was identified as the top homologous with 86% identity, as well as the top structural homolog (Protein Data Bank 3AML) (46) with a TM-score of 0.904 from the TM-align program (30). This SBEI structure was also chosen as the homolog for structure prediction by all six structural prediction tools that we used (see “Experimental Procedures”). The core and C-terminal regions of all six predicted structures agree quite well, however, there is variability at the N terminus of the protein likely due to poor sequence alignment between the first 55 N-terminal residues of maize SBEIIb and rice SBEI. Due to this variability observed at the N terminus, we decided to exclude these 55 residues before structural refinement by MD. In these MD experiments, the 1-TASSER (47, 35) structure was used as it was the only one of the six tools that permitted us to assign both deposited structures of SBEI (PDB code 3AML and 3AMK) (46) as templates for structure prediction.

In the 100-ns MD experiments, most of the global structural refinement occurs within the first 10 ns of the simulation with a root mean square deviation of ~5 Å at 10 ns compared with the starting structure. Subsequent to the first 10 ns of the simulation, there is very little global change and the conformation appears to be in a steady state (data not shown). The structure of SBEIIb at the end of the 100-ns simulation consists of three distinct domains: a central domain containing parallel β-strands arranged in a barrel surrounded by α-helices in a (β/α)8 arrangement, along with N- and C-terminal domains that each fold into an anti-parallel β-sandwich structure (Fig. 6A). All three phosphorylation sites are in disordered loop regions and are solvent exposed. Both Ser286 and Ser297 are close to the opening of the β-barrel on opposite sides.

Single phosphorylation at Ser286, Ser297, and Ser649, as well as double and triple phosphorylation are well accommodated with minimal change in global structure (data not shown). There are extensive salt bridge interactions between the phosphate group (−2 charge) and several Arg and Lys residues within the protein (Fig. 6, B–D). These salt bridges tend to form very early in the simulations (within the first 10 ns) and persist throughout the simulation. The phosphorylated Ser297 site was shown to be stabilized by interaction with Arg665 (Fig. 6C), part of a conserved Cys-containing region (Fig. 7). Generally, the pattern of salt bridges observed at each phosphorylation site was very consistent and was unaffected by phosphorylation at any of the other two sites.

**DISCUSSION**

Branching enzymes were the first class of proteins in the starch biosynthetic pathway shown to be directly regulated by protein phosphorylation (19). However, the role played by protein phosphorylation in regulating and coordinating the many enzymes of starch metabolism is not well understood. The aim...
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Phosphoamino acid analysis of HCl-digested maize SBEIIb indicates that the protein is phosphorylated at Ser residues, in common with previous studies with wheat endosperm SBEIIa and SBEIIb (19). Truncated forms of recombinant SBEIIb were helpful in determining the location of the most phosphorylated region of the protein (containing the Ser\(^{649}\) site), and bioinformatics was then employed to guide site-directed mutagenesis and biochemical analysis of likely phosphorylation sites. Of the number of probable phosphorylation sites predicted by bioinformatics, likely in vivo phosphorylation sites (i.e. those with scores of >0.9) were selected based on in vitro protein kinase assays using isolated amyloplasts as a source of protein kinase, and site-directed mutagenesis of specific Ser sites to Ala. Mutation of three Ser sites, Ser\(^{649}\), Ser\(^{286}\), and Ser\(^{297}\) to Ala each resulted in reduced phosphorylation of the mutant recombinant SBEIIb (Fig. 2), making them likely candidates for in vivo phosphorylation sites. Because phosphory-Ser\(^{649}\) site accounted for much of the \(^{32}\)P label on the native and recombinant proteins following incubation with [\(\gamma\)\(^{32}\)P]ATP and protein kinase, the loss of \(^{32}\)P label in Ser\(^{260}\) \(\rightarrow\) Ala and Ser\(^{297}\) \(\rightarrow\) Ala mutants could only be observed in double and triple mutants containing the Ser\(^{649}\) \(\rightarrow\) Ala mutation (Fig. 2D). Synthetic peptides containing each of the three putative phospho-Ser phosphorylation sites proved to be effective substrates for plastidial protein kinases at micromolar concentrations (Table 2). Confirmation that the three Ser sites are phosphorylated came from MS analysis of native SBEIIb and recombinant SBEIIb following treatment with protein kinase and ATP.

Synthetic peptides based on the three phosphorylation sites of SBEIIb and the recombinant protein all proved to be effective substrates for measuring and characterizing the plastidial protein kinase activities responsible for phosphorylation of SBEIIb. Amyloplast lysates used for phosphorylation experiments and subsequent purification were highly enriched from other subcellular contaminants (typically <0.3% cytosolic contamination and <0.2% contamination from other compartments determined using marker enzyme assays); it is therefore unlikely that protein kinases from compartments other than the amyloplast were responsible for phosphorylation of SBEIIb or the synthetic peptides. Two chromatographically distinct protein kinase activities were isolated from amyloplasts that showed distinct substrate preferences for the different synthetic peptides, and presumably the different phosphorylation sites on SBEIIb in vivo, and both protein kinase activities showed a dependence on Ca\(^{2+}\) ions. Although the elution profiles are consistent with the separation of two SBEIIb protein kinase activities, we cannot rule out the possibility that there may be more than one protein kinase associated with each peak. Identification of the proteins responsible for the kinase activities is a current focus of research. Ser\(^{297}\) is conserved among all plant SBEs (Fig. 7), suggesting a general regulatory role, whereas Ser\(^{286}\) is confined to the SBEII class. However, phosphorylation of Ser\(^{649}\) may have a more specialized function as this site appears only in some members of the cereal SBEIIb class. Interestingly, in other plant SBEIIs, Ser\(^{649}\) is, in some cases, substituted by Asp, a phospho-Ser mimic. The regions flanking the 11-amino acid sequence containing Ser\(^{649}\) are highly conserved in all classes of SBE. It is notable that the
Ser649 motif is completely absent from SBEI, again suggesting a distinct class function for this region.

Molecular modeling and MD studies produced a structure of SBEIIb that is very similar to SBEI from *O. sativa* L. (PDB codes 3AML and 3AMK), which is unsurprising given the high sequence and structural homology between the proteins, and the fact that this protein was used as a template in modeling. SBEIIb contains the three structural and functional domains that have been found to be highly conserved among SBEs within the glycoside hydrolase family 13 (GH13); namely the central catalytic domain, the N-terminal carbohydrate binding module, and the C-terminal domain involved in substrate specificity (11, 12). The three confirmed phosphorylation sites at Ser286, Ser297, and Ser649 are all on the surface of the protein in highly disordered regions (Fig. 6) making them highly accessible to protein kinases. Although phosphorylation at these sites did not result in a major conformational change in SBEIIb, phosphorylation will alter the surface charge of the enzyme that could be critical in modulating protein-protein interactions. This is particularly pertinent given previous observations that protein phosphorylation stimulates the formation of multienzyme complexes involving the interaction of SBEIIb with other enzymes of starch synthesis (19–24). The formation of stabilizing salt bridges between the phosphate groups and basic residues within disordered loop regions could play an important role in constraining these loops, possibly orientating them for binding interactions. Additionally, Ser286 and Ser297, which are located on opposite ends of the central catalytic β-barrel could be ideally positioned to interact with a glucan substrate, a process that would undoubtedly be affected by phosphorylation. Studies of the wheat enzyme suggest that enzyme activity is modulated by protein phosphorylation (19). Mutation of Ser286 → Ala or Ser297 → Ala caused a significant (more than 4-fold) loss in catalytic activity, reinforcing the critical role of these Ser residues in enzyme function. Overall, our modeling and MD studies show that SBEIIb has the typical fold of SBEs within the GH13 family and demonstrates that phosphorylation at the three identified sites does not cause a major structural change but could play an important role in modulating protein interactions and catalysis.

The results presented constitute direct evidence of specific phosphorylation sites on one of the enzymes associated with the pathway of starch biosynthesis. Given the centrality of the latter to the determination of yield in cereals, and the resolution of two, site-specific, protein kinase activities, this knowledge provides new possibilities for the targeted manipulation of a major metabolic pathway with potential application for the food and non-food industries in which starch is widely exploited.

FIGURE 6. Structure of SBEIIb obtained from homology modeling and molecular dynamics simulations. The images were rendered in VMD version 1.9.1 (43) and captured at the end of the 100 ns MD simulation. The protein is represented as a ribbon, whereas all labeled residues are represented as CPK. Panel A shows unmodified SBEIIb and highlights the location of the three identified phosphorylation sites: Ser286, Ser297, and Ser649 along with the three conserved structural and functional domains. These domains, namely, the central (β/α)₈ structure along with N- and C-terminal anti-parallel β-sandwich structures are colored yellow, blue, and red, respectively. Within the central (β/α)₈ domain are four highly conserved regions that are important for catalysis, namely: (I) residues 376–381, (II) residues 443–452, (III) residues 498–505, and (IV) residues 562–570 are colored cyan, orange, magenta, and green, respectively. Panels B–D illustrates that phosphorylation at positions Ser286 (PhSer286), Ser297 (PhSer297), and Ser649 (PhSer649) are stabilized by salt bridge interactions involving Arg and Lys residues.
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