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Identification of the Uridine-Binding Domain of Sucrose-Phosphate Synthase[1]

Expression of a Region of the Protein that Photoaffinity Labels with 5-Azidouridine Diphosphate-Glucose

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The uridine diphosphate-glucose (UDP-Glc) binding domain of sucrose-phosphate synthase (SPS) was identified by overexpressing part of the gene from spinach (*Spinacia oleracea*). Degenerate oligonucleotide primers corresponding to two tryptic peptides common to both the full-length 120-kD SPS subunit and an 82-kD form that photoaffinity labeled with 5-azidouridine diphosphate-glucose (5-N_3UDP-Glc) were used in a polymerase chain reaction to isolate a partial cDNA clone. Comparison of the deduced amino acid sequence of spinach SPS with the sequences of potato sucrose synthase showed that the partial cDNA included one region that was highly conserved between the proteins. Expression of the partial cDNA clone of SPS in *Escherichia coli* produced a 26-kD fusion protein that photoaffinity labeled with 5-N_3UDP-Glc. Photoaffinity labeling of the 26-kD fusion protein was specific, indicating that this portion of the SPS protein harbors the UDP-Glc-binding domain. Isolation of a modified peptide from the photolabeled protein provided tentative identification of amino acid residues that make up the uridine-binding domain of SPS.

Nucleotide diphosphate sugars supply the carbohydrate moiety for a number of important biosynthetic reactions. In the source leaves of higher plants, the penultimate step in the synthesis of Suc is the glucosyl transfer reaction, UDP-Glc + Fru-6-P → Suc-6-P + UDP. The enzyme that catalyzes this reaction, SPS (EC 2.4.1.14), has been purified to near homogeneity from spinach (*Spinacia oleracea*) (Salvucci et al., 1990), wheat (Salerno et al., 1991), and maize (Bruneau et al., 1991), and the sequences of cDNA clones for maize (Worrell et al., 1991) and spinach (Klein et al., 1993) SPS have been reported. Immunochemical studies with the spinach enzyme first showed that SPS is comprised of 120-kD subunits (Walker and Huber, 1989). Purification of the protein and photoaffinity labeling of the active site with the substrate analog 5-N_3UDP-Glc confirmed the identity of the catalytic subunit and established that the protein is homodimeric (Salvucci et al., 1990).

In sink tissues, SS (EC 2.4.1.13) catalyzes a reversible glucosyl transfer from UDP-Glc to Fru. However, in contrast to SPS, SS is thought to be primarily responsible for Suc hydrolysis rather than its synthesis (Avigad, 1982; Hawker, 1985). SS has been purified from mung bean seeds (Delmer, 1972), potato tuber (Pressey, 1969), and maize kernels (Su and Preiss, 1978). The protein is homomeric, made up of two to four 88-kD subunits. The sequence of cDNA clones encoding SS have been determined for several species including potato (Salanoubat and Belliard, 1987) and maize (Werr et al., 1985). Computer-generated alignment of the deduced amino acid sequences of SPS and SS from maize revealed only weak homology except in the region between SPS amino acids 488 and 686 (Worrell et al., 1991). The shared sequence identity in this region was 25%.

Little is known about the structure of nucleotide sugar-binding domains. Affinity-labeling studies of UDP-Glc pyrophosphorylase from potato tuber indicate that lysyl residues from at least four distinct regions of the protein may participate in binding or catalysis (Kazuta et al., 1991, 1993). UDP-Glc pyrophosphorylase and many other enzymes that use nucleotide sugars as substrates exhibit a K_m(UDP-Glc) value in the micromolar region. In contrast, the K_m(UDP-Glc) values for SPS and SS are in the 1 to 10 mM range (Avigad, 1982), suggesting that the structure of the UDP-Glc-binding domains of SPS and SS may be similar. In the present study, a partial cDNA encoding a 26-kD portion of SPS near the N terminus was expressed in *Escherichia coli*. The 26-kD SPS fusion protein photoaffinity labeled with 5-N_3UDP-Glc, indicating that it harbored the UDP-Glc-binding domain. The specific region of the 26-kD protein that was covalently modified by 5-N_3UDP-Glc was identified.

MATERIALS AND METHODS

Chemicals

[β-^{32}P]5-N_3UDP-Glc was synthesized as described by Drake et al. (1989). Acrylamide and other reagents used for

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Abbreviations: IPTG, isopropylthiogalactoside; 5-N_3UDP-Glc, 5-azidouridine 5′-diphosphate-Glc; PCR, polymerase chain reaction; SPS, Suc-P synthase; SS, Suc synthase.

electrophoresis were from Bio-Rad (Richmond, CA)[2]. Except where indicated below, all other reagents were from Sigma Chemical Co. or GIBCO BRL.

Purification and Assay of SPS

SPS was purified from field-grown spinach (*Spinacia oleracia*) leaves as described previously (Salvucci et al., 1990). The preparations used in the present study had specific activities of 67 to 100 units mg^{-1}. SPS was assayed at 30°C by monitoring UDP formation in a two-stage assay (Salvucci et al., 1990). Assays were conducted in duplicate.

Production of Polyclonal Antibodies

Polyclonal antibodies to purified leaf SPS or to a 26-kD portion of spinach SPS expressed in *E. coli* (see below) were prepared in BALB mice as described by Vogeli et al. (1990). The immune serum produced against purified SPS was affinity purified by adsorption to and desorption from the 120-kD full-length or the 82-kD truncated SPS polypeptides (see below) bound to an Immobilon-P poly(vinylidenedifluoride) membrane (Millipore, Waltham, MA) as follows. The 120- and the 82-kD polypeptides were separated by SDS-PAGE and transferred to a poly(vinylidenedifluoride) membrane as described previously (Salvucci et al., 1992). Polypeptides were localized by staining the margins of the transfer membrane with Coomassie blue according to the manufacturer's instructions. Following excision from the blot, the unstained strips containing the 120- or 82-kD polypeptides were incubated first for 2 h in 5% BSA to block unreacted sites and then for 4 h in immune serum. Affinity-purified anti-SPS antibodies were eluted at pH 2.5 as described by Smith and Fisher (1984).

Immunoprecipitation

Purified SPS from spinach leaves was incubated at 23°C in 25 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, and 6 mM DTT with either affinity-purified anti-SPS serum or preimmune serum. After 10 min, SPS activity was assayed as described above.

Western Blot Analysis

Western blot analysis was conducted on leaf extracts and purified SPS. Soluble leaf extracts were prepared as described previously (Salvucci et al., 1990). Protein in the extract was precipitated by addition of methanol to 80% (v/v), and the protein pellet was dried under N_2. The dried protein pellets were resuspended under denaturing conditions (Salvucci et al., 1990) and heated for 2 min at 100°C. The polypeptides in the resuspended pellet were separated by SDS-PAGE in 8 or 11% polyacrylamide minigels and then transferred from the gel to nitrocellulose (Crafts-Brandner et al., 1990). The nitrocellulose blot was incubated overnight with affinity-purified anti-SPS (120 kD) antibodies, and the SPS polypeptide was visualized using an alkaline phosphatase-conjugated secondary antibody system.

Peptide Profiles and Sequence Analysis

For analysis of SPS polypeptides, purified SPS was transferred to Immobilon-P as described above. Immobilon-P strips harboring the 120-kD polypeptide were blocked with 0.1% PVP-40 in methanol as described by Bauw et al. (1989) and then were incubated at 37°C with 1 μg of L-1-tosylamide-2-phenylethyl chloromethane ketone-treated trypsin. After 4 h, the strips were rinsed sequentially with 80% formic acid and H_2O to elute tryptic peptides (Bauw et al., 1989). The rinses were combined and were dried under vacuum. Peptides in the mixture were resuspended in 0.1% TFA and separated by reverse-phase HPLC on a 4.6- × 250-mm ISCO (Lincoln, NE) C-8 column (Salvucci et al., 1992).

Isolation of a Partial cDNA by PCR

A PCR-dependent strategy for isolating a cDNA for spinach SPS was designed based on the amino acid sequences obtained from digestion of immobilized spinach SPS with trypsin (Klein et al., 1993).

Expression of SPS Partial cDNA in *E. coli*

To permit the expression of a 26-kD fragment of spinach SPS in *E. coli*, the PCR-derived partial cDNA described above was cloned into pET T7 expression plasmid (Novagen, Madison, WI) essentially as described by Klein and Salvucci (1992). An *Nco*I restriction site was created at the 5′ end of the sense primer and an *Eco*RV restriction site at the 5′ end of the antisense primer. The primers were: sense primer 1s, 5′-GCGACCATG GGT TTT GAT GAG ACC GAT CTT C-3′, and antisense primer 1a, 5′-CAC GAT ATC CAT CTC AGT AGG TTC ACC ATA A-3′. The underlined portion of the primers represent nontemplate nucleotides that created the restriction sites necessary for cloning.

PCR reaction conditions were as described by Klein and Salvucci (1992) except the annealing temperature was 59°C. Following thermal cycling, amplified DNA sequences were analyzed on agarose gels, gel purified, and blunt-end cloned into an SK plasmid. Recombinant clones were sequenced (Klein and Salvucci, 1992) to examine for the presence of errors (mutations) generated by the Taq polymerase. The SPS cDNA was subcloned into pET 11d plasmid, and *E. coli* cells (BL21-DE3) containing the recombinant pET-SPS plasmid were induced with 0.4 mM IPTG when culture densities reached an A_{600} of 0.7 to 1.0. The protein product generated by this construct contains a single N-terminal Met not encoded by SPS and a 21-amino acid C-terminal fusion peptide encoded by the pET vector. The amino terminal Met was created by the *Nco*I restriction site, whereas the C-terminal fusion peptide was necessary to keep the SPS fusion polypeptide soluble (see text).

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Photoaffinity Labeling and Isolation of Photolabeled Peptides

Photoaffinity labeling with 5-N_3UDP-Glc was performed as described previously (Salvucci et al., 1990); the concentrations of photoprobe and UDP-Glc have been noted in Figure 6. For isolation of photolabeled peptides, approximately 5 mg of the 26-kD SPS fusion protein, isolated from *E. coli* inclusion bodies, was photolabeled at 4°C with 70 μM 5-N_3UDP-Glc in a total volume of 1 mL. After two rounds of photolabeling, unincorporated probe was removed by dialysis against 2 L of 20 mM NH_4HCO_3 at 4°C. After 4 h, 8 M urea was added to the dialysate to make the solution 2 M urea. The photolabeled protein was digested at 25°C by incubation for 12 h with 30 μg of L-1-tosylamide-2-phenylethyl chloromethane ketone-treated trypsin. Peptides generated by proteolysis were separated on an AX-300 (ISCO) anion-exchange HPLC column as described by Wise et al. (1987). Peak fractions containing radioactivity were further fractionated by reverse-phase HPLC (Salvucci et al., 1992). Fractions containing both peptides and radioactivity were subjected to sequence analysis at the University of Kentucky Macromolecular Facility using an Applied Biosystems 477A pulse liquid protein sequencer with on-line 120A PTH identification.

In separate experiments, two other procedures were used to isolate photolabeled peptides. In one experiment, photolabeled protein was digested with 25 units of *Staphylococcus aureus* V8 protease, and the photolabeled peptides were affinity purified by metal ion chelate chromatography at pH 8.0 as described by Salvucci et al. (1992), before isolation by ion-exchange and reverse-phase HPLC. In another experiment, photolabeled tryptic peptides were affinity purified by metal ion chromatography at pH 3.0 and then separated by reverse-phase HPLC. Edman degradation analysis of the isolated peptides showed that the same region of the protein was modified in all three experiments.

Miscellaneous

Protein was determined by the method of Bradford (1976) using BSA as a standard. Computer alignment of the amino acid sequences was performed using the CLUSTAL program (Higgins and Sharp, 1989).

RESULTS

In a previous study, SDS-PAGE analysis showed that polypeptides of 120 and 82 kD were the major Coomassie blue-staining polypeptides after purification of SPS from spinach leaves (Salvucci et al., 1990). Both of these polypeptides were photoaffinity labeled with 5-N_3UDP-Glc, a substrate analog that covalently modifies SPS at the UDP-Glc-binding site (Salvucci et al., 1990). As a starting point for identifying the uridine-binding domain, the relationship between the 120- and 82-kD peptides was investigated. Antibodies prepared against the SPS preparation recognized both the 120- and the 82-kD polypeptides on western blots (Fig. 1). Affinity-purified antibodies to the 120- and 82-kD polypeptide inhibited SPS activity (data not shown), and both antibodies recognized the 120- and the 82-kD polypeptides

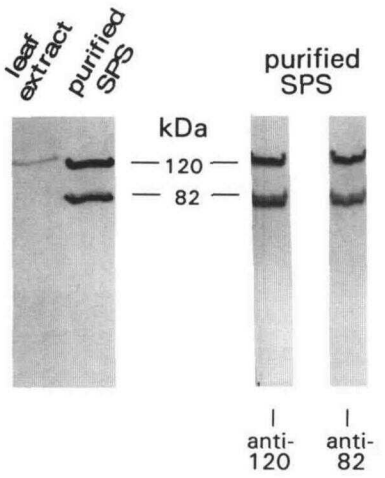

Figure 1. Immunological cross-reactivity of the 120- and 82-kD SPS polypeptides. Western blots of leaf extracts and purified SPS probed with polyclonal antibodies to SPS (lanes 1 and 2). Western blot of spinach SPS probed with affinity-purified antibodies to the 120- (anti-120, lane 3) or 82-kD (anti-82, lane 4) polypeptides.

on western blots of purified SPS. In contrast, only a single polypeptide of 120 kD was recognized when freshly prepared spinach leaf extracts were probed with anti-120- or anti-82-kD polypeptide antibodies. Lower molecular mass polypeptides, including one at 82 kD, were observed if crude spinach leaf extracts were incubated for >10 min at room temperature in the absence of protease inhibitors (data not shown). The results showed that the 82-kD polypeptide in partially purified preparations of SPS was immunologically related to SPS and suggested that it is a truncated form of the enzyme generated by proteolysis during purification.

Tryptic peptide mapping of the 120- and the 82-kD polypeptides by reverse-phase HPLC provided additional evidence that the 82-kD polypeptide is a truncated form of SPS that harbors the UDP-Glc-binding domain (Fig. 2). The chromatograms of the 120- and 82-kD polypeptides exhibited extensive similarity, except in the region from 20 through 28 min, where additional peptides were present in the chromatogram of the longer, 120-kD polypeptide. The similarity in the tryptic maps indicated that the 120- and 82-kD polypeptides share a common primary structure.

Definitive evidence for a common primary structure was provided by Edman degradation sequence analysis of internal peptides from two corresponding regions in the 120- and 82-kD chromatograms. Peptides with retention times of 28 and 31 min (designated T-28 and T-31) had identical amino acid sequences (see Fig. 3 for sequences). However, N-terminal sequence analysis of the 120- and 82-kD polypeptides by Edman degradation was unsuccessful, suggesting that their N termini were blocked.

The partial amino acid sequence of SPS facilitated cloning of the SPS gene using PCR. Sense and antisense oligonucleotide primer pools corresponding to specific portions of the T-31 and T-28 peptides were synthesized (Klein et al., 1993). First-strand cDNA was used as a template, and primer pools corresponding to peptide T-31 in a sense and peptide T-28

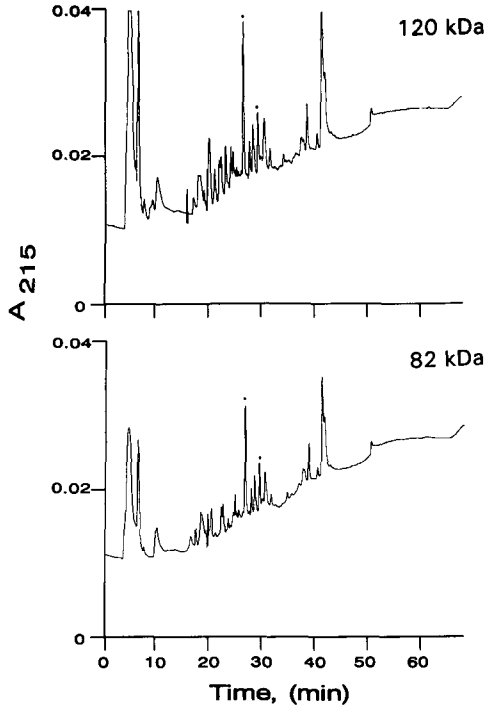

Figure 2. Reverse-phase HPLC of tryptic peptides from the 120- and 82-kD polypeptides. Peptides from corresponding regions in the 120- and 82-kD chromatograms (marked with an asterisk) had identical amino acid sequences.

in an antisense orientation directed the synthesis of a single 561-nucleotide PCR product from a region of overlap between the 120- and 82-kD SPS forms. The 561-nucleotide PCR product was then used to isolate a full-length cDNA clone for spinach SPS (Klein et al., 1993).

The deduced amino acid sequence of the 561-nucleotide PCR product and its position in the SPS open reading frame is shown in Figure 3. The identity of the amplification product as encoding for SPS was confirmed by comparison of the deduced amino acid sequence of the cDNA with tryptic peptides T-28, T-31, T-22 (Fig. 2), and cyanogen bromide fragments C-19 and C-34 from purified spinach SPS (shown overlined in Fig. 3). The deduced amino acid sequence of the PCR-derived cDNA was identical with that of the full-length cDNA of spinach SPS except for the incorrect identification of Ser^{236} as an Asn during peptide sequencing of tryptic fragment T-28. The deduced amino acid sequence of the full-length cDNA clone of spinach SPS was 54% homologous with the amino acid sequence of maize SPS (Klein et al., 1993).

In a search of the current gene banks for sequences homologous to the deduced amino acid sequence of spinach SPS, weak (i.e. 14.6%) overall homology was observed with SS. However, there were regions of much higher amino acid sequence homology between SPS and SS (data not shown). One of these regions was near the C terminus of the 26-kD SPS fusion protein. As shown in Scheme 1, the primary structure of spinach SPS in the region V176 to M243 was 34% identical (:) and 50% similar (.) or identical with potato SS. Spinach SPS is 87% identical with maize SPS in this region (Klein et al., 1993).

In a previous study, truncated forms of SPS, including the 82-kD form, were found to still bind 5-N_3UDP-Glc, a photoaffinity analog of UDP-Glc (Salvucci et al., 1990). For this reason, we investigated whether the region of SPS encoded by the 561-nucleotide cDNA, a region present in the 82-kD form, was active or could bind an analog of UDP-Glc. Cloning of the 561-nucleotide cDNA into a pET T7 expression vector permitted the production in *E. coli* of the region of SPS from F50 to M243. When *E. coli* cells were incubated for 3 h with 0.4 mM IPTG, a target protein of 26 kD accumulated in cells harboring the recombinant pET-SPS plasmid (Fig. 4). This polypeptide represented greater than 20% of the total cellular protein in *E. coli* based on Coomassie blue-staining intensity. Western blot analysis with anti-SPS immunoglobulin G confirmed that the 26-kD polypeptide was related immunologically to SPS (Fig. 4). Anti-SPS immunoglobulin G also reacted with a 52-kD polypeptide that may represent a dimeric form of the SPS polypeptide. Antibodies directed against the 26-kD SPS polypeptide recognized a 120-kD polypeptide in spinach leaf extracts (Fig. 4).

When expressed in *E. coli*, nearly all of the SPS protein was localized in inclusion bodies. Proteins in the inclusion bodies could be solubilized by treatment with 8 M urea but precipitated upon dialysis. Inclusion of a 21-amino acid, C-terminal fusion peptide of vector residues (DDPAANKARKEAELAAATAEQ) was sufficient to keep most of the SPS fusion protein soluble after dialysis. Following dialysis and centrifugation of inclusion body precipitate, the SPS fusion polypeptide represented greater than 90% of the solubilized protein from the inclusion body. The solubilized SPS fusion protein was enzymically inactive.

Photoaffinity labeling with [β-^{32}P]5-N_3UDP-Glc was conducted with the 26-kD SPS fusion protein synthesized in *E. coli*. As shown in Figure 5, the 26-kD SPS polypeptide was covalently modified by photoaffinity labeling with [β-^{32}P]5-N_3UDP-Glc. ^{32}P incorporation into the 26-kD SPS polypeptide was dependent on photolysis with UV light, and there was no photolabeling of a 26-kD polypeptide in cells that were not induced with IPTG. However, a 48-kD polypeptide was photolabeled with N_3UDP-Glc in solubilized inclusion bodies from both induced and noninduced cells. This polypeptide is probably a constitutively expressed *E. coli* protein

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SPS 176VVLISLHGLIRGENMELGRDSDTGGQVKYVVELARALGSMPGVYRVDLLTRQVSAPGVDW--SYGEPTEM243
        ::..: ::    ::. ::   :::::: :...   ::          ..: : .   :.:        : .
SS  283VVILSPHGYFAQENV-LGY-PDTGGQVVYILDQVPALER-------EMLKR-IKEQGLDIIPRILIVTRL342
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Scheme 1.

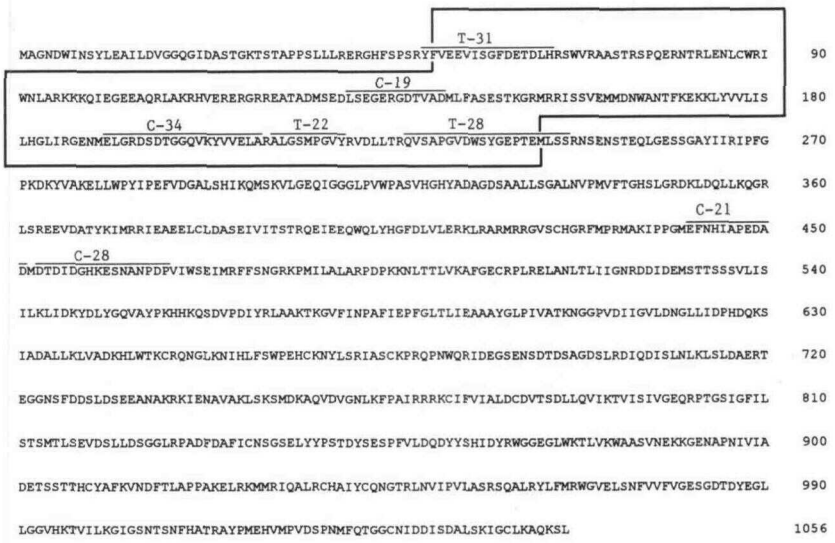

Figure 3. The position of amino acids encoded by the PCR-derived partial cDNA (block) in the deduced sequence of spinach SPS. The full-length polypeptide starts at the first Met codon of the large open reading frame. Segments corresponding to tryptic (T-22, T-28, T-31) and cyanogen bromide (C-19, C-21, C-28, C-34) peptide sequences are marked by the line above the the amino acid sequence.

that photolabels with much greater efficiency than the 26-kD SPS fusion protein.

When determined as a function of the 5-N_3UDP-Glc concentration, photoincorporation of 5-N_3UDP-Glc into the 26-kD SPS fusion protein exhibited hyperbolic kinetics (Fig. 6A). The apparent K_d for binding was 250 μM, compared with 74 μM for the 120-kD SPS polypeptide in protein isolated from leaves (Salvucci et al., 1990). Competitive binding experiments showed that the substrate UDP-Glc protected against photolabeling of the 26-kD SPS fusion protein fragment (Fig. 6B). These data indicate that photolabeling of the SPS fusion protein was specific and occurred at the UDP-Glc-binding site.

To identify the portion of the protein modified by 5-N_3UDP-Glc, the 26-kD SPS fusion protein was photolabeled with 5-N_3UDP-Glc and digested with trypsin, and the peptides were separated by ion-exchange HPLC. Reverse-phase HPLC analysis of the four radioactive peaks separated by ion-exchange HPLC showed that only one of these peaks

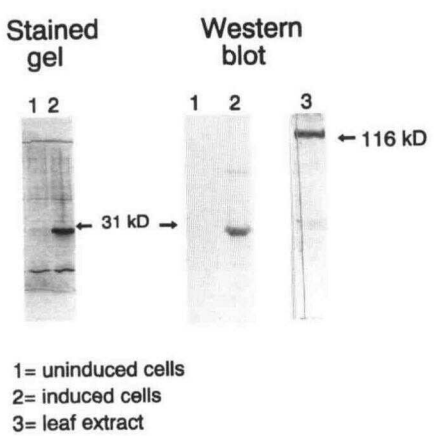

Figure 4. Synthesis of a 26-kD fragment of SPS in *E. coli*. Stained gel: Coomassie blue-stained SDS-PAGE gel of *E. coli* polypeptides. Polypeptides in lanes 1 and 2 were synthesized in cells containing recombinant SPS26 pET plasmids either before (lane 1) or after (lane 2) the addition of IPTG. Western blot: Western blot of *E. coli* proteins that were synthesized in cells containing recombinant SPS26 pET plasmids either before (lane 1) or after (lane 2) the addition of IPTG and then probed with affinity-purified anti-SPS antibodies. Lane 3 is a western blot of polypeptides in a spinach leaf extract probed with antibodies directed against the cloned 26-kD SPS fusion protein.

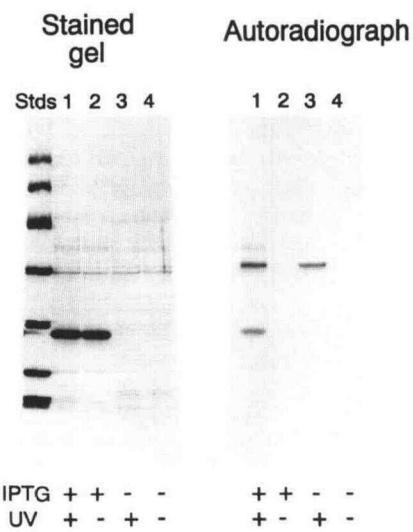

Figure 5. Photoaffinity labeling of the cloned SPS fusion protein with 5-N_3UDP-Glc. Polypeptides in solubilized inclusion bodies from *E. coli* cells harboring the recombinant SPS26 pET plasmids either after (lanes 1 and 2) or before (lanes 3 and 4) induction with IPTG were separated by SDS-PAGE after incubation with 50 μM [β-^{32}P]5-N_3UDP-Glc. Reactions were either kept dark or photolyzed with UV light. Coomassie blue-stained gel shows the positions of molecular mass standards for 97.4, 66, 45, 31, 21, and 14.4 kD.

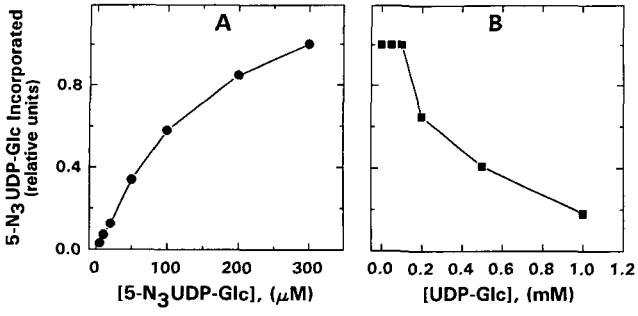

Figure 6. Concentration dependence of photoaffinity labeling of the 26-kD SPS fusion protein with [β-^{32}P]5-N_3UDP-Glc (A) and protection by UDP-Glc against photolabeling (B). Protein (15 μg) from solubilized inclusion bodies was photolyzed in the presence of the indicated concentrations of [β-^{32}P]5-N_3UDP-Glc (A) or in the presence of 50 μM [β-^{32}P]5-N_3UDP-Glc and the indicated concentrations of UDP-Glc (B). Incorporation was determined from densitometer scans of the autoradiographs and is expressed as the integrated peak area (absorbance units mm^{-1}). The K_d value was determined from a plot of 5-N_3UDP-Glc incorporation versus [5-N_3UDP-Glc].

contained photolabeled peptides and the rest contained primarily breakdown products from the photoprobe (data not shown). Most of the radioactivity in the peptide-containing peak eluted with the void volume of the reverse-phase column. Loss of radioactivity from photolabeled peptides is a common occurrence with reverse-phase HPLC, reflecting the lability of the *N*-glycosidic and photoinserted bonds (for discussion, see Salvucci et al., 1992). Beyond the void volume, there was a shoulder on the main radioactive peak that corresponded to single peptide peak in the chromatogram (Fig. 7). The amino acid sequence of the peptide was QVSAPGVDWSYGEPTE, which corresponds to the region Q227 to E242 of the deduced amino acid sequence of SPS (Fig. 3). The identical peptide was obtained when photolabeled tryptic peptides were affinity purified by metal ion chelate chromatography before reverse-phase HPLC (data not shown). Also, digestion of the photolabled fusion protein with *S. aureus* V8 protease generated a photolabeled peptide, L208-E239, that was from the same region of the protein.

DISCUSSION

Some limited conclusions can be drawn about the structure of SPS and the location of the UDP-Glc-binding domain from comparative analysis of the peptide profiles, immunological properties, and photoaffinity-labeling characteristics of the 120- and the 82-kD SPS polypeptides. For example, western blot analysis of crude leaf extracts showed that the native subunit of SPS is a 120-kD polypeptide (Fig. 1; Walker and Huber, 1989). That the 82-kD polypeptide is a form of SPS was indicated by (a) immunological cross-reactivity with the 120-kD polypeptide and (b) primary sequence homology between internal peptides from the 120- and 82-kD polypeptides. Because the photoaffinity-labeling characteristics of the 120- and 82-kD SPS polypeptides were identical (Salvucci et al., 1990), the binding site for UDP-Glc must occur on the region of overlap between the 120- and 82-kD forms of SPS.

Isolation of a partial cDNA from a region of SPS common to the 120- and the 82-kD polypeptides facilitated refinement of the structure of SPS. In addition to permitting the isolation of a full-length cDNA for spinach SPS (Klein et al., 1993), the partial cDNA in *E. coli* made it possible to identify the UDP-Glc-binding domain of SPS. The 26-kD portion of SPS encoded by the partial cDNA photoaffinity labeled with the substrate analog 5-N_3UDP-Glc. Photoaffinity labeling of the 26-kD fusion protein had a K_d(N_3UDP-Glc) of 250 μM and was blocked by the authentic substrate UDP-Glc. These results indicated that the photoprobe specifically modified a UDP-Glc-binding site on the SPS fragment and suggest that the region from F50 to M243 harbors the UDG-Glc-binding domain of SPS. Although the K_d(N_3UDP-Glc) for photolabeling of the 26-kD fusion protein was about 3-fold higher than the value for photolabeling of the 120-kD polypeptide of purified SPS, it was still 6-fold less than the K_m(UDP-Glc) for SPS. The higher K_d(N_3UDP-Glc) for the fusion protein compared to the native enzyme suggests that regions outside of F50 to M243 may also participate in binding UDP-Glc.

Unfortunately, there are no consensus binding motifs for nucleotide diphosphate sugars. SPS exhibits little homology with UDP-Glc pyrophosphorylase and other UDP-Glc-binding proteins, except SS. In agreement with homology comparisons, the K_m(UDP-Glc) values for SPS and SS are in the 1 to 10 mM range, whereas other enzymes that use nucleotide sugars as substrates exhibit K_m values in the micromolar range. Comparison of the primary structures of SS and SPS shows that homology between SPS and SS is particularly high in three regions, one of which corresponds to a region encompassed by the 26-kD SPS fusion protein. This region is 87% conserved between spinach and maize SPS (Klein et al., 1993).

Isolation and sequence analysis of the photolabeled peptide

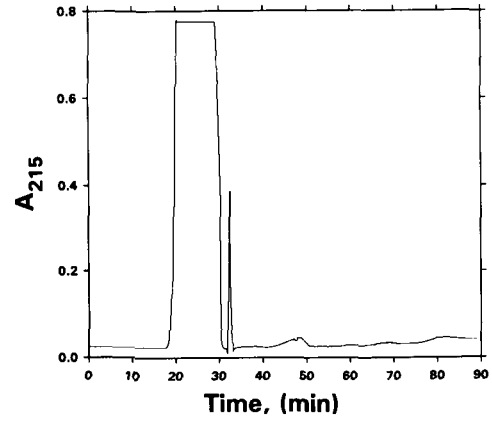

Figure 7. Reverse-phase HPLC of photolabeled tryptic peptides from the 26-kD SPS fusion protein. Tryptic peptides were generated from the 26-kD SPS fusion protein after photoaffinity labeling with [β-^{32}P]5-N_3UDP-Glc and then were separated by anion-exchange HPLC prior to isolation by reverse-phase HPLC.

made it possible to establish that the region from Q227 to E239 is in proximity to the 5 position of the uridine ring. This region of the protein is adjacent to E175 to L212, one of the most highly conserved between SS and SPS, and contains two residues, G232 and D234, that are conserved. In addition, 10 of the 13 residues between Q227 to E239 are conserved between maize and spinach SPS.

It may seem surprising that there is not more homology between SPS and SS in the uridine-binding domain; however, in general, the most highly conserved regions of nucleotide-binding proteins are those that bind the nucleotide phosphates rather than the nucleotide base. For example, the most highly conserved region among purine nucleotide triphosphatases is the P-loop, a Gly-rich region involved in phosphate binding (Saraste et al., 1990). The x-ray crystal structures of adenylate kinase (Muller and Schulz, 1992) and the *ras* oncogene product p21 (Pai et al., 1990) show that the nucleotide phosphates form numerous contacts with the protein, particularly with residues in the P-loop. In contrast, interactions between the nucleotide base and the protein are far fewer in number. As a result, the energy associated with base binding is much lower. A limited number of contacts between the protein and the nucleotide base may permit variability among nucleotide-binding proteins in the structure of the base-binding subsite. Variability in primary structure would explain why a consensus uridine-binding domain has not been uncovered.

To overcome losses caused by lability of the photoinserted probe, relatively large amounts of SPS fusion protein were required for the isolation of the covalently modified peptides. Because of the large amounts of starting protein needed for photolabeled peptide isolation and the relatively low abundance of SPS in plant tissues (Walker and Huber, 1989; Salvucci et al., 1990), it would be difficult to obtain sufficient protein to conduct similar experiments with SPS from leaves. Hence, recombinant technology provided a source of this key regulatory enzyme for affinity-labeling experiments. Further studies with N_3UDP-Glc and other affinity-labeling reagents using recombinant full-length and various truncated and mutated forms of SPS should make it possible to identify and modify the regions of SPS involved in catalysis, substrate-binding, covalent modification, and allosteric regulation.

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