Rubisco Small and Large Subunit N-Methyltransferases

BI- AND MONO-FUNCTIONAL METHYLTRANSFERASES THAT METHYLATE THE SMALL AND LARGE SUBUNITS OF RUBISCO

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is methylated at the α-amino group of the N-terminal methionine of the processed form of the small subunit (SS), and at the ε-amino group of lysine-14 of the large subunit (LS) in some species. The Rubisco LS methyltransferase (LSMT) gene has been cloned and expressed from pea and specifically methylates lysine-14 of the LS of Rubisco. We determine here that both pea and tobacco Rubisco LSMT also exhibit "N-methyltransferase activity toward the SS of Rubisco, suggesting that a single gene product can produce a bifunctional protein methyltransferase capable of catalyzing both "N-methylation of the SS and "N-methylation of the LS. A homologue of the Rubisco LSMT gene (rbcMT-S) has also been identified in spinach that is closely related to Rubisco LSMT sequences from pea and tobacco. Two mRNAs are produced from rbcMT-S, and both long and short forms of the spinach cDNAs were expressed in Escherichia coli cells and shown to catalyze methylation of the α-amino group of the N-terminal methionine of the SS of Rubisco. Thus, the absence of lysine-14 methylation in species such as spinach is apparently a consequence of a monofunctional protein methyltransferase incapable of methylating Lys-14, with activity limited to methylation of the SS.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) experiences several types of post-translational modifications during the expression, import, and assembly of the protein. The large subunit (LS) is encoded by a plastid gene and is transcribed and assembled into Rubisco holoenzyme within the stroma of the chloroplast. Mass spectral and amino acid sequence analysis of peptides prepared from Rubisco has demonstrated that the LS is processed to the mature form by removal of the N-terminal Met-1 and Ser-2 residues and acetylation of Pro-3 (1, 2). In addition the LS from many species, but not all, contains a trimethyllysyl residue at Lys-14 (2, 3, 4). The small subunit (SS) of Rubisco is also post-translationally modified. The polypeptide is post-translationally imported into chloroplasts and processed by a stromal processing peptidase that removes the targeting presequence. The resultant N-terminal methionine residue of the processed SS is subjected to N-methylation (5) prior to assembly with the LS into the holoenzyme.

Rubisco LSMT genes, cDNAs, and polypeptides have been characterized from pea and tobacco (6, 7). The methylation of Lys-14 of the LS is catalyzed by AdoMet:Rubisco LS N-methyltransferase (Rubisco LSMT, Protein Methyltransferase III, EC 2.1.1.127) (6). The amino acid sequences for tobacco and pea Rubisco LSMT show significant identity and similarity, and both proteins contain several copies of an imperfect leucine-rich repeat (7). Lys-14 methylation has been detected in many plant species (pea, tobacco, tomato, soybean, cucumber, petunia, pepper, and cowpea); however, in some species (spinach, wheat, and corn) methylation of lysine-14 does not occur (2, 4).

The observation that LS polypeptides from some species are methylated at Lys-14, whereas methylation is not detected in species such as spinach is a curious observation. Preliminary studies indicated that spinach contained LSMT-like DNA sequences. In this study we report a homologue of Rubisco LSMT spin from spinach that encodes an enzyme that methylates the SS of Rubisco (Rubisco SSMT). In addition, pea and tobacco Rubisco LSMT are demonstrated to catalyze α-methylation of the processed form of Rubisco SS. Thus, pea Rubisco LSMT is a bifunctional enzyme that catalyzes methylation of both the α-amino group of the SS and the ε-amino group of Lys-14 of the LS. This is the first reported eukaryotic "N-methyltransferase and of a bifunctional methyltransferase that methylates both α- and ε-amino groups.

EXPERIMENTAL PROCEDURES

Plant Material—Spinach (Spinacea oleracea L. cv. Melody) plants were cultured in ProMix® soil medium in a greenhouse at approximately 20 °C with a natural light photoperiod during the winter season (Lexington, Kentucky).

Cloning and Sequencing of rbcMT-S cDNAs—rbcMT-S cDNAs were obtained by reverse transcription-polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE). Total RNA was isolated from spinach leaves using Trizol (Life Technologies, Inc.). Reverse transcription-PCR was performed by reverse-transcription of 5 μg of total spinach RNA with an oligo d(T)17 primer. The first-strand cDNA product was amplified by PCR with Taq polymerase (Life Technologies, Inc.) using degenerate oligonucleotide primers to conserved peptide sequences between pea (6) and tobacco (7) Rubisco LSMTs. The forward

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primer (SF-8, 5'-CGA TGG GTA TTT GGA ATT CTG AGA TCA AGG GC) included an EcoRI site and corresponded to the amino acid sequence WAFGILRSA. The reverse primer (SR-2, 5'-GCC CAA GGC CAA GAT CAT TTA GAC TCA) primed cDNA synthesis in the mid-coding region and was followed by poly(dC) tailing as described previously (9). The cDNA-tailed products were amplified using a nested primer (SR-5, 5'-GCC GCA GAT CAT TTG GAG AGC TAC CTG GGC) and 250 ng RNase A (Sigma). The PCR products were digested with Sall and XbaI, gel purified, and cloned into Bluescript II KS(+) vector (Stratagene), and designated pS25.

RACE was performed to obtain 5' cDNA sequences as described above except that the reverse primer (SR-3, 5'-ACT GTA ACC AGA AGA ACT GTA AGC ACT ACA) primed cDNA synthesis in the region where N-terminal truncated forms of S-38 and S-40 were cloned into pET-23d for expression and have been submitted to GenBank (accession numbers AF071544 and AF071545) for sequencing and designated pS2.

To five independent clones were chosen for sequencing from each of the above constructs. Both strands of each clone were sequenced by the dideoxy chain termination method (10) using Sequenase (U.S. Biochemical Corp.) and [32S]dATP (NEN Life Science Products) with M13 reverse and 40 primers. In addition, 18–27-mer oligonucleotides were synthesized from DNA sequence obtained and used as primers for additional sequencing.

Both full-length S-38 and S-40 cDNAs (accession numbers AF071544 and AF071545, respectively) were obtained by ligation of clones pS2 and pS25 into pS38 or pS40 based on restriction sites within the overlapping regions. N-terminally truncated forms of S-38 and S-40 were produced by deletion of the putative chloroplast transit peptide through PCR with primers designed to remove the first 43 amino acids and replace Cys-44 with a Met. Both full-length and N-terminally truncated forms of S-38 and S-40 were cloned into pCR-Script Direct SK(+) vector (Stratagene) for sequencing and designated pS2.

Isolation and DNA Gel Blot Analysis of the rbcMT-S Gene—The rbcMT-S gene (accession number AF071543) was cloned by PCR. Spinach nuclear DNA was isolated using Floraclean (Bio101, Inc.). Approximately 100 ng of the nuclear DNA was amplified by PCR with Tq polymerase (Life Technologies, Inc.) using a forward primer (SF-1, 5'-AGA AGC TTC ACC ATG GCA ATG TTA TTC ATC ATC) and a reverse primer (SR-1, 5'-AGT TGG CAT TTA TTC ATG GAC TAC). The PCR product was cloned into pCR-Script SK(+) for sequencing.

The spinach rbcMT-S cDNA was transcribed with T7 RNA polymerase in the presence of [32P]ATP (800 Ci/mmol) to produce a radiolabeled antisense transcript. The radioactive probe (~100,000 cpm) was precipitated with 5 μg of RNA and 0, 2.5, 10, or 20 μg of total RNA isolated from mature spinach leaves. A heterologous RNA (10 μg of maize mitochondrion RNA) was included as a control. The RNAs were dissolved in 10 μl of hybridization buffer (80% formamide, 40 μl Pipes-NaOH, pH 6.4, 400 μm NaCl, 1 μM EDTA), denatured, and allowed to hybridize at 45 °C overnight. The duplexes were digested by addition of 0.3 ml of RNase digestion buffer (10 ml Tris-HCl, pH 7.6, 300 μM NaCl, 5 μM EDTA) with 200 units of RNase T1 (Roche Molecular Biochemicals) and 250 ng RNase A (Sigma). The duplexes were digested for 1 h at 30 °C, and the reaction terminated by addition of 100 μg of proteinase K and SDS to a final concentration of 0.6% and incubation for 30 min at 37 °C. The protected fragments were electrophoresed on 6% polyacrylamide gels with 8 μl urea. Gels were dried and autoradiographed.

Rubisco SSMT Activity Assays, Immunoblot, and Purification Procedures—Full-length and N-terminally truncated forms of S-38 and S-39 were cloned, expressed, and purified. Bacterial cells were cultured at 37 °C for 3.5 h in 5 ml of LB broth with 50 μg/ml carbenicillin and 35 μg/ml chloramphenicol. After induction with 0.5 mM IPTG, cultures were incubated for 2.5 h at 25 °C or 37 °C, and cells were collected by centrifugation at 5000 × g for 5 min at 4 °C. The cells were washed twice with deionized water and resuspended in 100 μl of buffer A (50 mM Tris-HCl, pH 8.2, 5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and fraction at ~80 °C. The activity of Rubisco SSMT was determined in the thawed cell lysates as described previously (11). Rubisco SSMT activity was determined by incubation of pLysS cell lysates, or purified S-38 and S-40, with lysates from spinach chloroplasts (12). Reactions contained 2.5–5 μCi of [3H-methyl]AdoMet (5–88 Ci/mmol), 10–20 μg of chloroplast plastid lysate protein, and either 10 μg of protein from pLysS cell lysates, or 1 μg of purified S-38 or S-40 protein. After incubation at 30 °C for 30 min, reactions were terminated by addition of SDS-PAGE sample preparation buffer, and samples were electrophoresed on 15% acrylamide gels. After transfer of proteins to a PVDF membrane, radioactivity was visualized with a 3H PhosphorImager screen (Molecular Dynamics model 425, Sunnyvale, CA), and polypeptides were visualized by staining with Coomassie Brilliant Blue R-250. Alternatively, the PVDF-blotted proteins were subjected to Immunoblot analysis using antibodies prepared against pea Rubisco LSMT or S-38 protein.

Full-length and N-terminally truncated forms of S-38 and S-40 were purified from pLysS cell lysates. pLysS cells were cultured at 37 °C, and inclusion bodies were harvested after cell lysis and washed twice with buffer A before dissolving in 6 M urea. Cell debris was removed by centrifugation (5 min, 12,000 × g), and the remaining supernatant was dialyzed against buffer A for 4 h. The dialysate contained 1–2 mg/ml purified protein that was used directly in activity assays and to elicit antibodies (Charles River PharmServices, Southbridge, MA).

RESULTS

Isolation of rbcMT-S cDNAs—Degenerate oligonucleotide primers were designed to highly conserved sequences of pea and tobacco Rubisco LSMT and were used to amplify a 786-bp cDNA fragment from total spinach leaf RNA. DNA sequence analysis of the 786-bp fragment indicated that the spinach cDNA had extensive sequence identity with pea and tobacco LSMT. A complete cDNA sequence of the spinach cDNA was obtained by 5' and 3' RACE of spinach leaf RNA (13).

Amplification of 5' and 3' coding sequences was performed by RACE. PCR amplification resulted in the identification of two 5' products (836- and 848-bp fragments). The 848-bp product had a 12-bp insertion relative to the 836-bp fragment. The 5' RACE products and the initial PCR product amplified with LSMT-specific primers had complete sequence identity in the regions that overlapped, except for the 12-bp insertion. Amplification of 3' coding sequence was performed by RACE, and a single 761-bp PCR product was obtained. Complete cDNA sequences have been assembled from these overlapping clones of the rbcMT-S and have been submitted to GenBank as accession numbers AF071544 and AF071545.

The spinach rbcMT-S cDNAs encode polypeptides of 495 (S-40) and 491 amino acids (S-38) with predicted molecular masses of approximately 55 kDa, similar to the masses of pea (55.0 kDa) and tobacco (56.0 kDa) Rubisco LSMT (Fig. 1). In addition, the deduced amino acid sequence of the spinach cDNAs included five imperfect copies of a leucine-rich repeat region (14), similar to pea and tobacco LSMT (7). The deduced amino acid sequence from the spinach rbcMT-S cDNAs shows 60 and 62% identity with the amino acid sequences of pea and tobacco Rubisco LSMT, respectively (Fig. 1).

Transcription and Expression of rbcMT-S—A 3.1-kilobase genomic clone that included the entire coding region of the spinach rbcMT-S gene was obtained and sequenced (GenBank accession number AF071540). The spinsch rbcMT-S gene included six exons and five introns and has similar genomic organization with the tobacco Rubisco LSMT gene (rbcMT-T). DNA gel blot analysis indicated that the rbcMT-S gene is a single copy gene in spinach (data not shown).

The 12-bp insertion in the 5' end of the rbcMT-S-S-40 cDNA corresponds to the 3' 12 nucleotides of intron II (Fig. 2). RNAase protection studies were performed to analyze the relative expression of the S-38 and S-40 mRNA in spinach leaves. A 390-nucleotide subclone of the S-40 cDNA was prepared that spanned exons 3 and 4 (Fig. 3A). The S-40 mRNA (long form) would protect the entire 390-nucleotide region, whereas S-38 mRNA (short form)
would protect 306- and 72-nucleotide fragments. A 460-nucleotide antisense probe was produced by transcription with T3 RNA polymerase (Fig. 3B, lane 1) and hybridized to total spinach leaf RNA. Digestion with RNase A and T1 revealed that the S-40-specific fragment (390 nucleotides) and the S-38-specific fragments (306 and 72 nucleotides) were present in approximately equal abundance (lanes 3–5). Control reactions included omission of spinach leaf RNA (lane 2) or substitution of heterologous RNA (maize mitochondrial RNA; lane 6), and these reactions failed to protect any fragments. Both S-40 and S-38 mRNAs were not detectable in total RNA from spinach roots, stems, or flowers (data not shown). Thus, alternative 3' splice site selection produces two transcripts for the rbcMT-S gene that are expressed at similar abundance in spinach leaves.

Examination of the N-terminal sequence of S-38 and S-40 using ChloroP version 1.0 (15) identified a putative chloroplast processing site from Ser-41 to Cys-44 (Ser-Ile-Arg Cys), with exact identity to the chloroplast processing site for spinach nitrate reductase (16). The putative chloroplast targeting pre-sequence was removed, and the N-terminally truncated forms of spinach S-38 and S-40 were cloned in the pET23d expression vector and expressed in Escherichia coli. Induction with IPTG resulted in the synthesis of large amounts of protein in inclusion bodies. The recombinant protein was collected from lysed bacterial cells and purified. Catalytic activity was obtained after denaturation in 6M urea and renaturation by exhaustive dialysis.

Antibodies to pea Rubisco LSMT were not immunoreactive with any polypeptides from spinach leaf extracts or bacterially expressed forms of S-38 and S-40. Therefore, polyclonal anti-
bodies were prepared against the purified N-terminally truncated S-38 and used to detect spinach SSMT polypeptides. The SSMT antibody recognized a single polypeptide from spinach chloroplast lysates at a molecular mass expected for the processed form of S-38 (~50.6 kDa; Fig. 4). It was not possible to distinguish between S-38 and S-40 protein forms on SDS-PAGE because the expected molecular mass shift is only 560 daltons. Duplicate protein blots were probed with antibody raised against Rubisco, which demonstrates that the single immunoreactive band observed with S-38 antibodies is not due to cross-reactivity to the LS of Rubisco (Fig. 4).

Spinach rbcMT-S Catalyzes Methylation of the α-Amino Group of the Processed Form of the SS of Rubisco—The presence of chloroplast-localized translation products for S-38 and S-40 with homology to both pea and tobacco Rubisco LSMT suggested that these proteins may have a different but related type of methyltransferase activity. Pea Rubisco LSMT catalyzes incorporation of tritiated methyl groups from [3H-methyl] AdoMet into Rubisco holoenzyme isolated from unmethylated species such as spinach (11). Truncated S-38 and S-40 proteins exhibited no methyltransferase activity toward purified spinach Rubisco or toward purified Rubisco from other plant species. A recent report of α-methylation of the N terminus of the processed form of the SS of Rubisco (5) led us to examine the possibility that the S-38 and S-40 cDNAs coded for an “N-methyltransferase for the SS of spinach Rubisco. N-terminally truncated S-38 and S-40 recombinant proteins were assayed for methyltransferase activity with spinach chloroplast lysates as a proteinaceous substrate (Fig. 5). Tritium radiolabel incorporation from [3H-methyl]AdoMet into spinach chloroplast polypeptides was analyzed by SDS-PAGE, electrophoretic transfer to PVDF membranes, and PhosphorImager analysis (Fig. 5A). Incorporation of radiolabel was detected into a region corresponding to the SS of Rubisco (Fig. 5A) that was dependent on the addition of the chloroplast lysate. A small amount of label incorporation was observed in the region of the SS in the absence of IPTG but was dramatically increased with the addition of IPTG and is a consequence of leaky expression of the pET-23d construct. No radiolabel was observed in pLysS cell lysates or chloroplast lysates alone. Both S-38 and S-40 were capable of catalyzing methylation of the SS (Fig. 5A). Highly purified S-38 was obtained from inclusion bodies and refolded as a soluble protein and was shown to exclusively catalyze transfer of radiolabel into the region corresponding to the SS of Rubisco (Fig. 5B). Similar results were obtained with purified S-40 (data not shown). These results suggest that a small pool of free SSs exists in the stromal fraction from spinach chloroplasts that function as a substrate for methylation. A pool of free SSs has been previously demonstrated in chloroplasts from Chlamydomonas (17) and peas (18).

Unequivocal demonstration that purified recombinant S-38 catalyzed SS methylation at the N-terminal Met of processed SS was documented by Edman degradation of the SS with simultaneous determination of radioactivity. During the first cycle of Edman degradative sequencing, 92% of the radiolabel incorporated into the small subunit region of the PVDF blot was released (Fig. 6). The amino acid residue in this cycle did not correspond to any of the standard amino acids but migrated as a unknown with a retention time just slightly greater than N-diphenylurea, similar to that reported for methylated Met in other work (5). The next 11 amino acid residues corresponded exactly to the known N-terminal residues of the SS of spinach

![Fig. 3. Two forms of rbcMT-S mRNAs are produced as a result of alternative splice site selection.](image)

A. pS40XR0.4 was produced by cloning the 390-nucleotide EcoRI fragment of the S-40 cDNA into pBluescript SK+ . The clone spans exon III, the alternatively spliced region, and exon IV. A 460-nucleotide antisense probe was produced by transcription from the T3 promoter to the region, and exon IV. A 460-nucleotide antisense probe was produced by transcription from the T3 promoter to the region, and exon IV. A 460-nucleotide antisense probe was produced by transcription from the T3 promoter to the RI/m gene. B. RNase protection was performed to demonstrate the relative abundance of the long and short forms of rbcMT-S. The 460-nucleotide antisense probe (lane 1) was annealed to 0, 2.5, 10, or 20 µg of total RNA from mature spinach leaves (lanes 2–5, respectively) and digested with RNase A and RNase T1. The 390- and 306-nucleotide fragments are similar, indicating that the long and short forms are approximately equivalent in abundance. Omission of spin (RNA (lane 2)) or the substitution of a heterologous RNA (maize mitochondrial RNA; lane 6) resulted in complete sensitivity of the probe to RNase. The migration of a radiolabeled DNA size ladder (250-bp ladder, Life Technologies, Inc.) is shown on the right margin.

![Fig. 4. Immunodetection of translation products from rbcMT-S in spinach chloroplasts.](image)

Isolated intact chloroplasts from spinach leaves were lysed, and the stromal fraction was loaded on SDS-PAGE (15% acrylamide) gels. After electrophoresis, proteins were electrophotographically transferred to PVDF membranes and either stained for several minutes with Coomassie Brilliant Blue R-250 (Stain) or developed with antibodies prepared against purified S-38 protein (Immunoblot). Lanes 1, 2, 4, 5, 7, and 8 are chloroplast lysates with total protein loads as indicated above the lanes. Lanes 3 and 6 are purified S-38 protein at the indicated levels. LS and SS identify the large and small subunits of spinach Rubisco, respectively.
Rubisco (19). Given that a thorough chemical and physical structural analysis of the N-terminal residue of the SS of spinach Rubisco has already identified this residue as N-methyl-methionine, these results demonstrate that the S-38 and S-40 cDNAs for the rbcMT-S gene encode enzymes that can methylate the N-terminal Met residue of the small subunit of Rubisco.

**Fig. 5.** S-38 and S-40 Expression-dependent incorporation of \(^{3}H\)-radiolabel from \(^{3}H\)-methyl|AdoMet into the small subunit of Rubisco in spinach chloroplast lysates. A, lysates (−15 μg of total protein) from induced (+IPTG) and non-induced (−IPTG) pLysS cells harboring S-38 or S-40 cDNAs cloned into pET23d expression vector were incubated in the presence and absence of spinach chloroplast lysates (−12 μg of total protein) and \(^{3}H\)-methyl|AdoMet (1.8 μM, 70–80 Ci/mmol). After incubation at 30 °C for 30 min, samples were electrophoresed on 15% SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The PVDF membranes were briefly stained with Coomassie Brilliant Blue R-250 and then destained with 100% methanol, washed with distilled water, and imaged for radioactivity. LS and SS refer to the locations of the large and small subunits of Rubisco, respectively. B, purified S-38 (−2 μg) was incubated in the presence and absence of spinach chloroplast lysates (−18 μg of total protein) and \(^{3}H\)-methyl|AdoMet (1.8 μM, 70–80 Ci/mmol) for 30 min at 30 °C. After electrophoresis and transfer, the PVDF membrane was stained and imaged as described above.

**Fig. 6.** Radiosequencing of the spinach rubisco SS. A spinach chloroplast lysate containing 100 μg of total protein was incubated with purified S-38 protein (10 μg) and \(^{3}H\)-methyl|AdoMet (1.8 μM, 70–80 Ci/mmol) for 30 min at 30 °C. The sample was electrophoresed on a 15% SDS-PAGE gel, and proteins were electrophoretically transferred to a PVDF membrane. The PVDF membrane was briefly stained with Coomassie Brilliant Blue R-250 and then imaged for radioactivity, and the protein band at the position of the SS of Rubisco was subjected to Edman degradative sequencing. During each cycle of sequencing a portion of the phenylthiohydantoin-derivatized amino acid pool was diverted prior to separation by high pressure liquid chromatography for determination of radioactivity. Amino acid residues identified are indicated, and all other residues were <5 pmol. LS and SS refer to the locations of the large and small subunits of Rubisco, respectively. The sequence obtained, identified at the bottom of the figure, is identical with the sequence reported for the SS of spinach Rubisco beginning with the second lysyl residue (19). The first residue released during sequencing, identified as a modified Met residue (indicated by an asterisk in the sequence bar graph) migrates slightly behind N-diphenylurea and is consistent with the sequence and recently reported mass spectroscopy identification of methyl-N-methionine as the N-terminal residue of the SS of spinach Rubisco (5).
Rubisco SSMT and LSMT

Pea and Tobacco Rubisco LSMT Exhibit SSMT Activity—
The high degree of sequence identity between spinach SSMT and pea and tobacco Rubisco LSMT as well as the evidence for SS methylation in these species (Ref. 5 and this work) suggest that the previously characterized pea and tobacco Rubisco LSMT may also possess SSMT activity. Purified pea chloroplast Rubisco LSMT and pea and tobacco recombinant LSMT bacteria were tested for N-methyltransferase activity toward the SS of spinach Rubisco. Pea LSMT efficiently methylated both SS and LS of Rubisco from spinach chloroplast lysates when assayed with high specific activity [3H-methyl]AdoMet (Fig. 7). Control reactions included cell lysates from uninduced (without IPTG) conditions and spinach chloroplast lysates alone and did not show radiolabel incorporation into the SS of spinach Rubisco. Earlier methylation experiments (3) were performed with purified Rubisco; however, assembled Rubisco is apparently a poor substrate for α-methyltransferase reactions. Tobacco Rubisco LSMT had not been previously demonstrated to catalyze LS methylation and in these assays only catalyzed radiolabel incorporation into the SS of spinach Rubisco. Thus, the pea, spinach, and tobacco enzymes catalyze α-amino group methylation of the SS, whereas the pea enzyme is bifunctional and also methylates Lys-14 of the LS.

**Discussion**

Rubisco is subjected to several forms of post-translational modifications, and these reactions include removal of two N-terminal amino acid residues and acetylation of Pro-3 of the LS (1, 2); α-methylation of Lys-14 of the LS (2); proteolytic cleavage of the SS by the stromal processing protease (20); and α-methylation of the Met-1 of the processed form of the SS (5). This paper demonstrates that the α-methylation reaction of the SS is catalyzed by enzymes in spinach and tobacco that are closely related to the previously characterized LS methyltransferase from pea. In addition, the pea enzyme has been shown to be bifunctional and possesses the capacity to methylate both the α-amino group of Met-1 of the SS and the ε-amino group of Lys-14 of the LS.

The *rbcMT-5* gene is present as a single copy in the spinach genome, and two splice forms of the mRNA are detected by PCR and by RNase protection. The two forms apparently result from alternative 3′ splice site selection: 1) DNA gel blot analysis indicated that the cDNA probe sequence is a single copy in the spinach genome; 2) sequence analysis of S-40 and S-38 cDNA clones revealed identical DNA sequence; and 3) RNase protection analysis detected both splice variants in similar abundance. S-40 transcripts include a 12-bp insertion that would result in an in-frame insertion of WVQQ into the polypeptide. The functions of the two forms of the spinach SSMT are currently unknown. Both long and short forms efficiently catalyzed SS methylation, but the previously characterized pea and tobacco methyltransferases lack the WVQQ insertion. It is possible that these proteins have different specificities and participate differently in various methylation reactions.

Both 3′ splice sites of the spinach SSMT gene are typical 3′ splice sites. The downstream (S-40) splice site has a typical 3′ splice boundary (CAG*GC) and compares well with the dicot consensus (YAG*GU) (21, 22). In addition, an upstream polypyrimidine stretch (UAUUUAUUU) is present, although the stretch extends 23 nucleotides upstream of the splice site and is perhaps somewhat further upstream than typical in plants. Possible branch sites exist at the A residues, which are 21 and 37 nucleotides upstream of the splice site. The short form (S-38) splice boundary (AAG*UG) includes the highly conserved AG preceding the splice site, a polypyrimidine stretch that extends upstream 10 nucleotides, and a possible branch site at 24 nucleotides upstream of the splice site.

A single immunoreactive polypeptide localized in the chloroplast was detected with polyclonal antibodies to S-38, but the small mass difference between the protein forms of S-38 and S-40 precludes determination of whether or not both of these proteins are present in vivo. Recombinant S-38 and S-40 proteins have SS methyltransferase activity. It is possible that other chloroplast proteins may be substrates for either S-38 or S-40; however, these may not be detected by methylation studies utilizing chloroplast lysates, because detection under these conditions depends on hypo-methylation of these polypeptides.

In the case of the SS of Rubisco, proteolytic processing by the stromal processing peptidase is a prerequisite for methylation of the N-terminal Met residue. The presence of unassembled SS and the requirement for proteolytic processing may be responsible for a small but easily detectable pool of methylatable SS in spinach chloroplast lysates.

Methylation of the SS of Rubisco is apparently widespread because N-methyl-Met was detected as the N-terminal residue in the SS of Rubisco from pea, corn, and barley (5). We have also detected N-methyl-Met as the N-terminal residue in the SS of tobacco Rubisco (data not shown). In addition, an enzymatic assay demonstrated that chloroplast lysates catalyzed methylation of bacterially expressed SS using AdoMet as a methyl donor (5).

The expression of a spinach homologue to Rubisco LSMT from pea and tobacco was initially anomalous, given that Lys-14 in the LS of spinach Rubisco is not methylated. Structural analyses of the methylation status of Lys-14 in the LS of spinach Rubisco unequivocally established this residue as unmodified (1, 4), and spinach Rubisco is an excellent *in vitro*
substrate for pea Rubisco LSMT (3, 11). Additional studies in our laboratory have demonstrated that the absence of methylation at Lys-14 in the LS of spinach Rubisco is consistent throughout the development of spinach plants and is not influenced by several environmental stresses.2 In addition, the gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial gene and coding sequences reported for spinach, pea, and tobacco Rubisco methyltransferases do not contain substantial

The bifunctionality of pea Rubisco LSMT is the first report of both an “N- and an ‘N-protein methylation activity in a single enzyme, and in addition this is the first report of a eukaryotic gene for a “N-methyltransferase. Methylation of E. coli ribosomal L11 protein involves both “N- and ‘N-methylation (23). Mutations in the prmA gene encoding the ribosomal protein L11 methyltransferase result in an under-methylated form of the L11 protein, which may be an indication of a similar prokaryotic bi-functional protein methyltransferase, although this has not been directly demonstrated. N-terminal methylation has been described for various amino acid residues in several proteins for both prokaryotic and eukaryotic organisms (24). The functional significance of this post-translational modification is obscure and has been speculated to be involved in processes as diverse as protection against proteolytic degradation (25, 26, 27), proper subcellular localization of proteins, and/or assembly of macromolecular structures (28). Protection against proteolytic degradation is a possible function because recent studies (34) as well as earlier reports (35, 36, 37) have described tubular connections between chloroplasts in vivo capable of allowing the exchange of molecules between chloroplasts, similar in function and form to bacterial pili.

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