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Postimport methylation of the small subunit of ribulose-1,5-bisphosphate carboxylase in chloroplasts

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Abstract Electron impact mass spectronomy analysis of the amino-terminal amino acid of the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase (Rubisco) showed that the amino-terminal methionine residue is post-translationally modified to *N*-methyl-methionine. Modification of the amino-terminal methionine residue was found in mature SSU proteins from the dicotyledonous plants pea and spinach as well as the monocotyledonous plants barley and corn. SSU methyltransferase is a soluble protein in the chloroplast stroma and accepts heterologously expressed non-methylated SSU as a substrate using S-adenosylmethionine as methyl-group donor. We show that this modification occurs after post-translational uptake of the precursor form of SSU into chloroplasts and processing to its mature size. This reaction represents a new step in the import and assembly pathway of Rubisco holoenzyme.

Key words: *Pisum sativum L*; Protein import; Protein methyl transferase; Post-translational modification

1. Introduction

The small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase (Rubisco) is encoded by a small multigene family in *Pisum sativum* [1–4]. The SSU-RNA is a major transcript of the nuclear DNA [5] and is translated in the cytoplasm as a larger precursor (preSSU) containing an amino-terminal transit peptide [6]. The presequence is both necessary and sufficient to promote productive targeting and translocation into chloroplasts. Already during or shortly after translocation into chloroplasts the presequence is cleaved off by the stromal processing protease to yield mature SSU [7]. Eight mature SSU polypeptides assemble in an chaperonin-aided process with an equal number of chloroplast-encoded large subunits (LSU) of Rubisco to form the holoenzyme in higher plants [8].

The *SSU* gene family is composed of at least five members, which are clustered on chromosome 5 within a region of four map units [9]. Two of the *SSU* genes have been localized to a single genomic clone [10]. The pea *SSU* genes cannot be divided into subfamilies based on nucleotide sequence homology because little nucleotide sequence divergence between the coding regions of the five pea *SSU* genes is observed, i.e. all genes encode an identical mature SSU [3,4]. The only amino acid replacements occur within the transit peptide region.

The primary structure of the mature SSU protein deduced from sequence analysis of the *preSSU* gene [1] was confirmed by protein sequencing of the purified SSU protein and proteolytic fragments of SSU [11]. Re-evaluation of the primary structure of purified mature SSU with modern protein chemical micromethods, such as matrix-assisted laser desorption ionization-time of flight mass spectronomy (MALDI-TOF/MS) showed that the amino-terminal methionine residue of the mature pea SSU is post-translationally modified to *N*-methyl-methionine. This post-translational and post-translocational modification of the amino-terminal methionine residue was also found in other mature dicot (spinach) and monocot (barley and corn) SSU proteins, suggesting that this modification is widespread in all SSU proteins.

2. Material and methods

2.1. Purification of SSU from pea, spinach, barley and corn

2.1.1. First method of purification. Ten-day-old pea seedlings or 8-day-old corn seedlings were harvested and ground with liquid nitrogen using a mortar and pestle. The plant powder was extracted with 3 ml of water per gram fresh tissue and centrifuged at $10\,000\times g$ for 20 min. Afterwards the extract was concentrated using microconcentrator tubes with 3 kDa cut-off filters. Further purification of SSU was performed by C-4 reversed-phase high performance liquid chromatography (HPLC) applying a standard water/acetonitrile gradient (1–60% acetontirile within 70 min). SSU-containing fractions were identified by MALDI-TOF/MS.

2.1.2. Second method of purification. For characterization of the N-terminal modification SSU was isolated from silica-sol purified intact pea chloroplast [12] (*Pisum sativum*, L., var. Golf). Chloroplasts were lysed on 10 mM HEPES–KOH (pH 7.6). A soluble protein fraction was recovered after centrifugation at $100\,000\times g$ for 1 h, and further centrifuged at $250\,000\times g$ for 16 h. Rubisco holoenzyme was recovered from the pellet fraction and proteins separated further by denaturing continuous electrophoresis in the presence of SDS using a 491 Prep Cell (BioRad, Hercules, USA) according to the manufacturer's protocol. Fractions containing SSU were pooled, dialyzed against water and used for further analysis.

2.2. MALDI-TOF/MS analysis

The purified SSU protein, complete protein digests and HPLC purified peptide fractions were subjected to MALDI-TOF/MS analysis using the Hewlett-Packard HP G2025A linear MALDI-TOF/MS system (Hewlett-Packard, Palo Alto, CA) equipped with a nitrogen laser (337 nm). Typically 1 μl of the SSU digests or HPLC peptide fractions were mixed with 2 μl of α-cyano-4-hydroxycinnamic acid as matrix

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Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase; SSU, small subunit of Rubisco; LSU, large subunit of Rubisco; preSSU, precursor form of SSU; MALDI-TOF/MS, matrix assisted laser desorption ionization-time of flight mass sprectronomy; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; EI-MS, electron impact mass spectronomy; DPTU, diphenylthiourea; SAM, S-adenosylmethionine

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(Hewlett-Packard, Palo Alto, CA), vacuum crystallized and analyzed. For the analysis of the mature SSU protein sinapic acid was used as matrix.

2.3. Protein sequencing

Automated protein sequence analysis of the intact SSU protein, peptides and α-methyl-methionine was carried out on the Hewlett-Packard HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA) using the routine 3.0 sequencing chemistry method according to the manufacturer's protocol.

2.4. Electron impact mass spectronomy analysis of the amino-terminal amino acid

About 3 nmol of the HPLC collected N-terminal peptide was subjected to protein sequence analysis and the amino-terminal phenylthiohydantoin amino acid (PTH-X) was manually collected. Prior to MS analysis the PTH-X was re-chromatographed by RP-HPLC as described above to remove salt components of the sequencer HPLC solvents. The electron impact mass spectronomy (EI-MS) spectra were obtained on an MAT-312 mass spectrometer (Varian, Palo Alto, CA). The electron impact mass spectra were recorded with electron energy of 70 eV at a temperature of the source block of 100°C.

2.5. Chloroplast isolation and protein import

Chloroplasts were isolated from pea plants 10 days after planting. Organelles were purified further on silicon-sol gradients as described before [12]. Chlorophyll was determined and chloroplasts equivalent to 80 μg chlorophyll were resuspended in 300 μl of import buffer (330 mM sorbitol, 2 mM ATP, 50 mM HEPES–KOH (pH 7.6), 3 mM $MgSO_4$, 10 mM $NaHCO_3$, 20 mM K-gluconat, 10 mM methionine, 2% BSA) in the presence of 3 μCi [^{14}C-methyl]S-adenosylmethionine (SAM, spec. act. 57 mCi/mmol) for 10 min at 4°C. The import reaction was initiated by the addition of 2 μg preSSU and allowed to continue for 20 min at 25°C. Aliquots (1/8 of the total reaction) were withdrawn at different time intervals and chloroplasts recovered by centrifugation (5000×g for 1 min). Chloroplasts were subsequently lysed in 200 μl of 10 mM HEPES–KOH (pH 7.6) and a soluble protein fraction containing SSU protein was prepared by centrifugation (150000×*g*, 10 min). Proteins were precipitated by the addition of an equal volume trichloroacetic acid (20% w/v) and washed three times. The final protein precipitate was dissolved in 10 μl of HCOOH and used to determine the incorporation rates by liquid scintillation counting. The final 50% of the import reaction was analyzed by SDS-PAGE and fluorography as described before [12]. PreSSU and SSU were expressed in *E. coli* BL21(DE3) cells using the pET vector system exactly as described before [13]. PreSSU and SSU were solubilized and denatured in 8 M urea and added to the reaction mixture. The final urea concentration did not exceed 60 mM.

2.6. In vitro methylation of SSU

A stromal extract was prepared from intact chloroplasts as described above. Methylation reactions were carried out in 50 mM HEPES–KOH (pH 7.6) in a final volume of 60 μl for 20 min at 25°C. Reaction products were analyzed by SDS-PAGE followed by fluorography.

3. Results and discussion

SSU from pea was purified either by reversed-phase chromatography from a total leaf extract or by SDS-PAGE from a stromal preparation of purified, intact chloroplasts. Amino-terminal protein sequence analysis showed an unknown PTH-amino acid derivative in cycle 1 which did not elute at the position of the expected PTH-methionine (retention time 15.1 min) but between DPTU and PTH-tryptophan (Fig. 1a). This PTH-derivative does not correspond to the known modified methionine residues PTH-methioninesulfoxide or methioninesulfone because these derivatives are more hydrophilic and would elute earlier in the HPLC chromatogram of the PTH-amino acid derivatives. Interestingly, amino-terminal protein sequence analysis of mature SSU proteins from another dicot species, i.e. spinach (not shown) and from two monocot species, i.e. barley and corn showed the identical modified amino acid at the amino-terminus (Fig. 1b,c), indicating that this post-translational modification is widespread throughout the plant kingdom and might be of general relevance to the function of Rubisco. Protein sequence analysis of the recombinant tobacco SSU protein [13] overexpressed in *E. coli* revealed the expected methionine residue at the amino-terminus (data not shown) indicating that this modification seems to be specific to the plant protein synthesis/post-translational modification machinery [14]. The amino-terminal sequencing of SSU from pea revealed the following amino acids Gln Val Trp Pro Ile - Gly Lys Lys Lys Phe Glu for positions 2–12, respectively, and was identical to those published previously [1–4,11]. Up

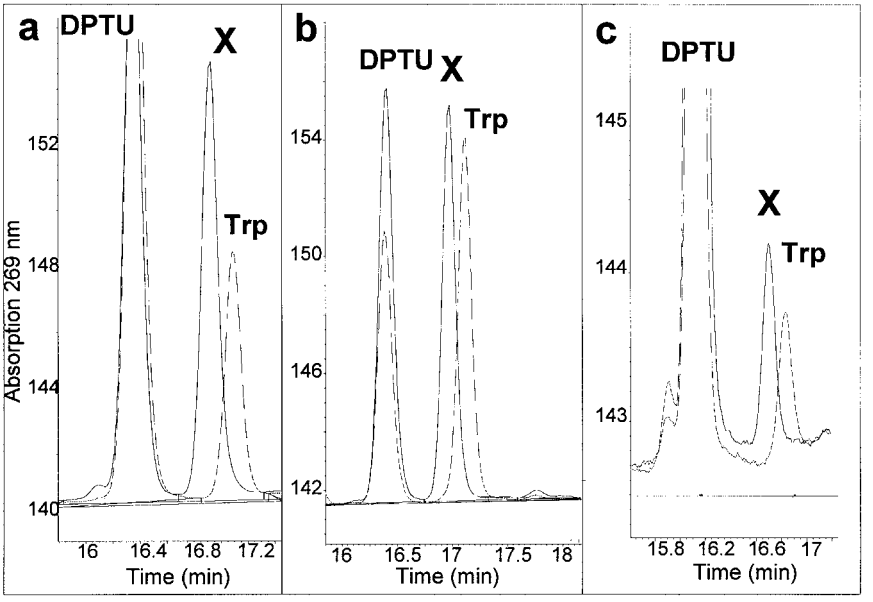

Fig. 1. Amino-terminal protein sequence analysis of mature SSU from pea (a), barley (b), and corn (c). Cycle 1 containing the modified amino acid X and cycle 4 containing Trp are overlayed. Diphenylthiourea (DPTU) is a by-product of Edman chemistry.

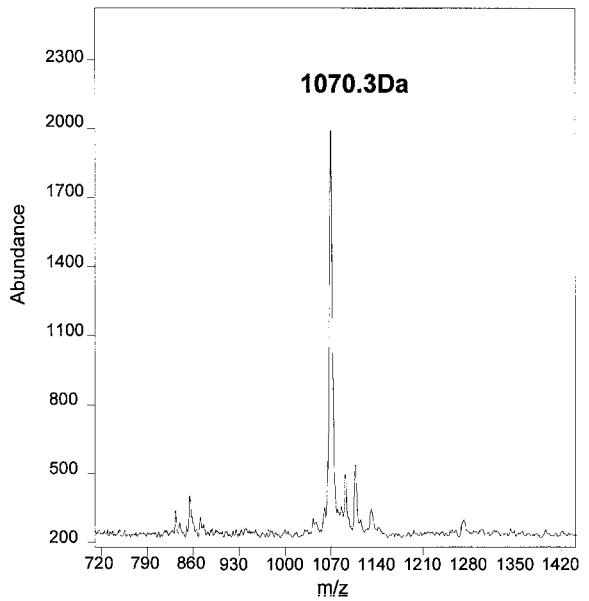

Fig. 2. Molecular mass determination of the HPLC purified amino-terminal tryptic SSU peptide by MALDI-TOF/MS analysis. Indicated mass represents peptide mass plus one proton (MH^+).

to 20% Gln was found in cycle 1 of the sequencing reaction, indicating that some proteolysis had occurred during the isolation.

Exact determination of the unusual modification of the amino-terminal amino acid residue of the mature SSU protein was achieved by EI-MS analysis of the collected amino-terminal PTH-amino acid from protein sequence analysis of the purified amino-terminal tryptic peptide. Mass determination of the amino-terminal peptide by MALDI-TOF/MS showed a molecular mass of 1070.3 Da (Fig. 2). Protein sequence analysis of the peptide resulted in the expected sequence X Gln Val Trp Pro Ile Gly Lys with the modified residue, indicated as X, at the amino-terminus. The molecular mass of the peptide containing a methionine at position 1 would be 1056.6 Da indicating that a mass difference of 14 Da is present in the amino-terminal methionine group which could be contributed by an additional methyl group. Control EI-MS experiments carried out with PTH-methionine (Fig. 3a) revealed, besides the mass of the entire molecule of 266 Da, characteristic mass losses of the amino acid side chain of 61 Da, corresponding to the loss of CH_2–S–CH_3 and of 74 Da, corresponding to the loss of CH_2–CH–S–CH_3 (Fig. 3a) due to McLafferty re-arrangement. Mass spectrometric analysis of

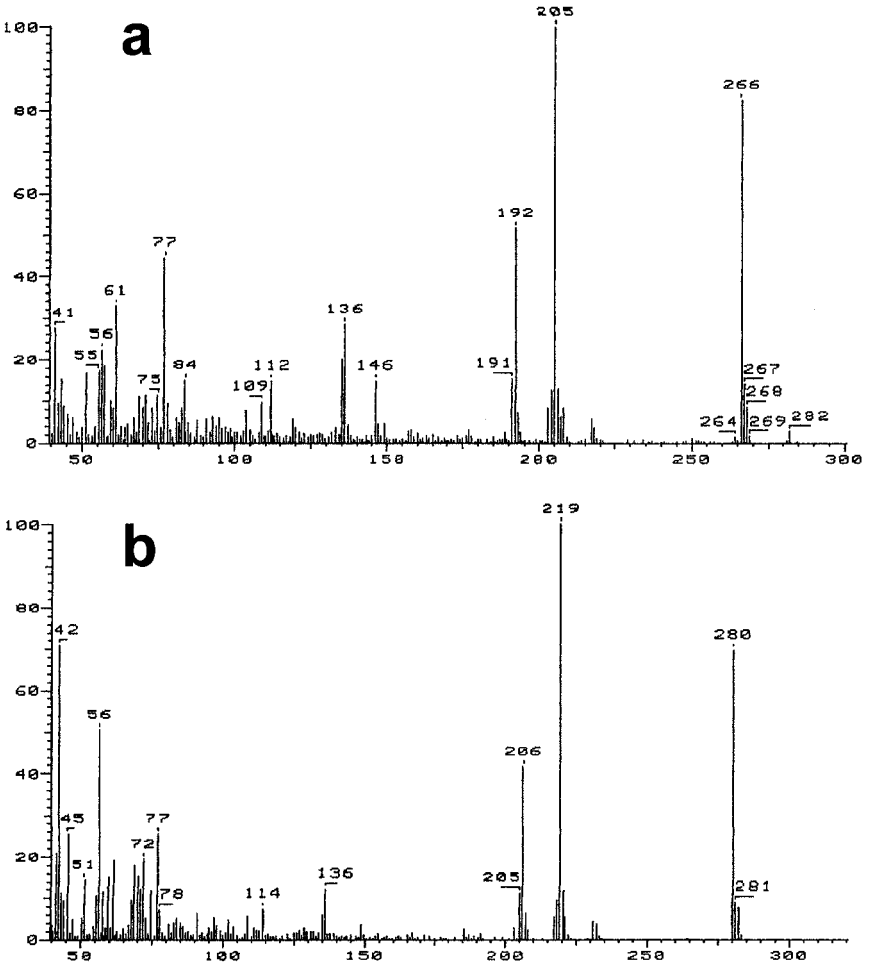

Fig. 3. Electron impact EI–MS analysis of (a) PTH-methionine as control and of (b) the collected amino-terminal PTH-X residue from pea SSU.

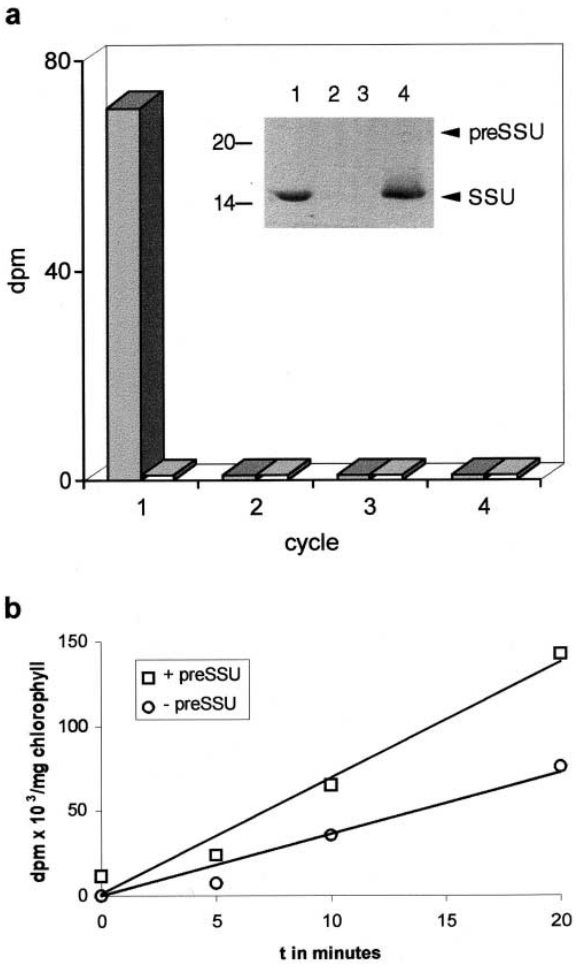

Fig. 4. Rubisco SSU is methylated by a stromal methyltransferase at the N-terminal methionine of the mature form. (a) A stromal extract was incubated either in the presence (left, darker bars) or absence (right, lighter bars) of 2 μg SSU and [^{14}C-methyl]SAM as methyl group donor. Edman degradation of SSU from a methyltransferase reaction was performed. The incorporation of radioactivity was determined by liquid scintillation counting. The inset shows a fluorogram of a methyltransferase reaction in presence (lane 1) or absence (lane 2) of SSU. Lane 3: 2 μg preSSU was added. Lane 4: Stromal proteins together with 10 μg of total chloroplast membranes were used in the presence of 2 μg exogenous SSU. Products were analysed by SDS-PAGE and fluorography. (b) Newly imported preSSU into chloroplasts stimulates methylation in intact chloroplasts. A standard import reaction was carried out either in the absence (○) or presence (□) of preSSU (2 μg) using chloroplasts equivalent to 40 μg chlorophyll in 200 μl of import buffer. Aliquots were withdrawn at the time points indicated and the incorporation of radioactivity into soluble proteins determined as outlined in Section 2.

the PTH-X amino acid resulted in the expected mass of the entire PTH-amino acid of 280 Da (Fig. 3b) suggesting the presence of an additional methyl group in the amino acid. The fragmentation pattern showed the same mass losses of 61 and 74 Da as obtained for PTH–methionine, indicating that the additional methyl group is not located next to the sulfur atom in the methionine residue but has to be located at the amine group as *N*-methyl modification. To exclude that the additional methyl group is located at α-C atom position of methionine α-methyl-methionine was sequenced. It was found that PTH-derivative was eluting very close after the DPTU peak in the sequencer HPLC chromatogram but before the PTH–X residue of the SSU amino-terminus (data not shown).

The occurrence of different polypeptide methyltransferases has been demonstrated in chloroplasts from either spinach [15] or pea [16]. In spinach chloroplasts the methyl residues were incorporated into the carboxyl groups of glutamic and aspartic acid of SSU and the light harvesting chlorophyll a/b-binding protein. Furthermore a lysine residue in the N-proximal region of LSU is modified to *N*-trimethyllysine in pea and a number of different plants [16,17]. Since the chemical analysis demonstrated the presence of a *N*-methyl-methionine at the N-terminus of SSU we wanted to know if chloroplasts are indeed capable of introducing the methyl group into SSU in vitro. The amino group of the N-terminal methionine is available for modification only after import of the SSU precursor into chloroplasts and processing to its mature form. Therefore the modification should occur concomitantly to the import and processing of preSSU in the organelle. In an initial approach to address this problem a soluble stromal extract was incubated in the absence or presence of mature SSU or preSSU using [^{14}C-methyl]SAM as methyl group donor. The addition of non-methylated SSU expressed in *E. coli* resulted in the incorporation of a ^{14}C-methyl group from SAM into the protein (Fig. 4a inset, lane 1). SSU in pea seems to be exclusively present as CH_3–SSU since we could detect the incorporation of methyl groups from SAM only upon addition of exogenous SSU substrate. (Fig. 4a inset, lane 2). Upon prolonged exposure of the X-ray film we could also detect labelled LSU (not shown). The absence of methylation in the presence of preSSU (Fig. 4a inset, lane 3) indicates that no further methylation sites are available or detectable in vitro in the protein except at the N-terminus of the mature form. The stromal extract used in the methyl transferase reaction is not able to process preSSU because the specific isolation procedures [18] to maintain processing competence were not used here. Addition of chloroplast membranes to the methyl transferase reaction did not increase the yield of methylated SSU, indicating that the SSU methionine methyl transferase is localized exclusively in the stroma (Fig. 4a inset, lane 4). These results clearly demonstrate the capacity of chloroplasts to post-translationally modify the SSU protein. To ensure that the incorporation of the methyl group into endogenous SSU also occurred in vitro at the N-terminus, a SAM methylated SSU was subjected to radiosequencing. The results in Fig. 4a clearly demonstrate that the radioactive methyl group is incorporated at amino acid position 1.

Next we wanted to know if protein methylation could also occur concomitantly with import and processing of preSSU in chloroplasts. Purified chloroplasts were 'preloaded' with [^{14}C-methyl]SAM for 10 min [19] after which preSSU was added. Import was allowed to continue 20 min, and aliquots were removed at different time points. A stromal extract was prepared and analyzed for the incorporation of radioactivity in soluble chloroplast proteins. A 2-fold increase of ^{14}C-methyl incorporation was observed when preSSU was present during the import reaction in comparison to a mock import reaction in the absence of preSSU (Fig. 4b). That SSU was indeed methylated was controlled by SDS-PAGE and fluorography (not shown). A higher rate of background methylation by SAM was detectable when intact organelles (not shown) were used than in the case of stromal extracts or lysed chloroplasts (compare Fig. 4a and b). In intact chloroplasts, meth-

ylation probably also occurs on these proteins targets, e.g. LSU and light-harvesting chlorophyll-binding proteins which were identified before [15,16] but with higher efficiency. From these data we conclude that methylation of newly imported preSSU occurs rapidly after the methylation site becomes accessible to methyltransferase after processing.

4. Conclusion

Protein methyltransferases can be divided into two major groups: (i) the generally reversible modification of carboxyl groups to form methyl esters and (ii) the generally irreversible methyl transfer to sulfur or nitrogen atoms [20]. The cellular function of the latter reaction is not fully understood at the moment, but generation of a new amino acid seems to influence the cellular role of the target protein. N-terminal modifications like the one reported here have been described only in a few instances. Firstly, methylation of amino-terminal amino acids has been described for ribosomal proteins in *E. coli* [20], but no functional implications were indicated for this modification. Secondly, *N*-methyl-methionine at the amino-terminus of the CheZ protein seems to play an important role in chemotaxis in certain bacteria [21]. To our knowledge, the transfer of a methyl group to amino-terminal amino acids has been described for prokaryotic organisms only, which would be in line with the prokaryotic origin of chloroplasts. Our findings clearly establish that this post-translational modification occurs in a eukaryotic context also namely in chloroplasts which are under nuclear control. At present we can only speculate on the physiological role of amino-terminal methylation, e.g. increased protein stability. This could be especially important in organelles such as mitochondria and plastids which import most of their protein constituents from the cytoplasm as precursor proteins. After processing inside the organelle post-translational and post-import modification might be required to guarantee a faithful life cycle of these proteins. The SSU has to be assembled with LSU subunit of Rubisco to form the L8S8 holoenzyme in pea and other higher plants. LSU itself is highly post-translationally modified, i.e. the N-terminus is acetylated [22], the N-proximal Lys is methylated [16,17] and the protein is carbamylated [23]. The enzymatic properties of the L8S8 holoenzyme form are likely to be influenced by these multiple post-translational modifications. Another functional reason could be that methylation of the N-terminal methionine in SSU inhibits a methionine amino peptidase, which otherwise might remove the N-terminal amino acid and subsequently induce proteolytic degradation of the protein [24,25]. Clearly more work is needed to elucidate the biological function of what seems to be a far more widespread phenomenon than previously recognized.

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