Co- and post-translational modifications in Rubisco: unanswered questions

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Received 3 October 2007; Revised 30 November 2007; Accepted 18 December 2007

Abstract

Both the large (LS) and small (SS) subunits of Rubisco are subject to a plethora of co- and post-translational modifications. With the exceptions of LS carbamylation and SS transit sequence processing, the remaining modifications, including deformylation, acetylation, methylation, and N-terminal proteolytic processing of the LS, are still biochemically and/or functionally undefined although they are found in nearly all forms of Rubisco from vascular plants. A collection of relatively unique enzymes catalyse these modifications, and several have been characterized in other organisms. Some of the observed modifications in the LS and SS clearly suggest novel changes in enzyme specificity and/or activity, and others have common features with other co- and post-translationally modifying enzymes. With the possible exception of Lys14 methylation in the LS, processing of both the LS and SS of Rubisco is by default an ordered process sequentially leading up to the final forms observed in the holoenzyme. An overview of the nature of structural modifications in the LS and SS of Rubisco is presented, and, where possible, the nature of the enzymes catalysing these modifications (either through similarity with other known enzymes or through direct enzymological characterization) is described. Overall, there are a distinct lack of functional and mechanistic observations for modifications in Rubisco and thus represent many potentially productive avenues for research.

Key words: Co-translational processing, dipeptidase, \textsuperscript{\textit{N}}-methyltransferase, methionine aminopeptidase, \textsuperscript{\textit{N}}-acetyltransferase, peptide deformylase, post-translational processing, Rubisco, stromal processing peptidase.

Introduction

Since its discovery as the principle enzyme responsible for photosynthetic fixation of carbon dioxide, a plethora of co- and post-translational protein modifications have been reported for Rubisco. Early findings describing proteolytic processing of the small subunit (SS) (Dobberstein \textit{et al.}, 1977; Chua and Schmidt, 1978; Robinson and Ellis, 1984) and carbamylation of the large subunit (LS) (Lorimer and Miziorko, 1980; Lorimer, 1981) sparked the development of several avenues of research which continue today. While the aforementioned modifications were quickly established as essential for critical aspects of Rubisco assembly and activity, many others that followed have not been resolved with regard to functional significance. Nevertheless, Rubisco has served as an excellent model for many aspects of co- and post-translational processing that have resulted in significant contributions to our understanding of the biochemical processes and enzymes responsible for these modifications.

Here an overview is presented of the co- and post-translational modifications in both the LS and SS that are currently known primarily for the vascular plant forms of Rubisco but for which information about the enzymes catalysing these modifications and/or their functional significance is limited.

N-terminal processing

By far the majority of protein modifications occur at the N-terminus of both the LS and SS of Rubisco, as indicated in Fig. 1. The amino acid sequences in these regions, particularly the first 3–4 amino acids, are also remarkably conserved across all vascular plant species (Fig. 2). N-terminal processing of the SS is rather well described, with the exception of methylation of the
The \( \alpha \)-amino group of Met1 in the mature form of the SS. In contrast, N-terminal processing of the LS is much more extensive and involves a number of steps which lead to the final mature form found in the Rubisco holoenzyme. It is worth noting that while the temporal relationship between events such as assembly and co- and post-translational processing of the SS and LS are defined at some steps, such as the removal of the SS transit sequence, the order of events depicted in Fig. 1 is strictly for convenience and not necessarily representative of their
et al. Fig. 1 for the LS which is known to be essential (Dirk
Deformylation is the only processing event indicated in
absolute prerequisite for all other N-terminal processing,
shown in Fig. 1A. It is very likely that this step is an
relationship to final holoenzyme assembly or other events
involved in the maturation of Rubisco.

LS deformylation
N-terminal processing of the LS begins with deformyla-
tion of N-formyl-Met1 by peptide deformylase (PDF) as
shown in Fig. 1A. It is very likely that this step is an
absolute prerequisite for all other N-terminal processing,
with the probable exception of Lys14 methylation.
Deformylation is the only processing event indicated in
Fig. 1 for the LS which is known to be essential (Dirk
et al., 2001; Hou et al., 2004, 2007). In plants, PDF exists
in two forms, PDF1A (formerly DEF1) and PDF1B
(formerly DEF2); both localized to chloroplasts and
mitochondria (Dirk et al., 2001; Dinkins et al., 2003).
PDF1A and PDF1B are partially functionally redundant
in vivo yet large differences exist between PDF1A and
PDF1B in polypeptide substrate specificity and catalytic
efficiency in vitro (Dirk et al., 2002). PDF1A and PDF1B
have similar activities against polypeptide mimics of the
LS N-terminus, but PDF1B is ~100-fold more active than
PDF1A using peptide mimics of the photosystem II D1
polypeptide. Treatment of intact plants with actinonin,
a potent peptide deformylase inhibitor, results in a rapid
reduction in photosystem II activity, D1 processing, and
assembly into photosystem II, ultimately leading to
photosynthetic bleaching and death (Hou et al., 2004).
However, overexpression of either PDF1A or PDF1B
results in nearly complete resistance to actinonin (Hou
et al., 2007). Given the widespread occurrence of PDF1A
and PDF1B in plants, the combined action of PDF
inhibitors and resistance conveyed by overexpression
creates an attractive combination of technologies for the
control of plant growth as well as a tool for use as a
selectable marker for genetic engineering of plants.

LS methionine and serine removal
Cleavage of methionine from the N-terminus of newly
translated polypeptides is perhaps one of the most
common and ubiquitous processing events. Methionine
removal is catalysed by methionine aminopeptidase
(MAP), a ubiquitous enzyme which is found throughout
the plant and animal kingdoms, as well as prokaryotic
and archaea kingdoms (Bradshaw et al., 1998; Falb et al.,
2006; Frottin et al., 2006). MAP activity is essential in
prokaryotic organisms and has been identified as an
anticancer target (Chang et al., 1989; Li and Chang,
1995; Vaughan et al., 2002; Selvakumar et al., 2006;
Sawanyawisuth et al., 2007). The role of MAP activity in
the chloroplasts is not defined beyond identification of at
least three forms from Arabidopsis thaliana targeted to
chloroplasts that are each capable of functional rescue of
Escherichia coli MAP mutants (Giglione et al., 2000).
MAP operates with polypeptide specificity primarily
dependent on the penultimate N-terminal residue (Arfin
et al., 1995; Bradshaw et al., 1998; Lowther and
Matthews, 2000). High activity is generally associated
with residues with small side chains such as alanine,
serine (as in the LS), and glycine. However, there is
a distinct influence of the antepenultimate position, with
substantial reductions in MAP activity associated with the
presence of a proline residue at this position (Frottin
et al., 2006). Thus, the sequence of the deformylated LS, Met-
Ser-Pro, would be predicted to retain the N-terminal
methionine residue. Therefore, as indicated in Fig. 1A,
the removal of Met1 and Ser2 could be a consequence of
a dipeptidase reaction where the two residues are removed
together, circumventing the necessity for MAP activity.
Alternatively, accepting the possibility that chloroplast-
localized MAPs could have unique enzymological and
specificity properties, the removal of Met1 and exposure
of Ser2 still necessitates further processing by some as yet
unidentified aminopeptidase with activity against N-
terminal serine residues. Regardless, these initial events
all lead to the manifestation of Pro3 as the N-terminal
residue of the LS prior to acetylation, as described below.
The lack of any definitive evidence for the exact manner
in which Met1 and Ser2 are removed, coupled with the
importance of MAP enzymes in other organisms, under-
scores the potential for future significant and novel
research contributions in the area of co-translational processing of the LS.

**LS acetylation**

Although N\(^{\text{\alpha}}\)-acetylation of Pro3 of the large subunit of Rubisco was unequivocally established nearly two decades ago (Houtz et al., 1989), both the enzyme responsible and the functional significance of the modification remain completely undetermined. As is the sequence of the N-terminus of the large subunit of Rubisco (Fig. 2), acetylation of Pro3 of the large subunit of Rubisco seems well conserved; this modification occurs not only in vascular plants but also both in a non-vascular plant (liverwort *Marchantia polymorpha*) and in a green alga (*Chlamydomonas reinhardtii*) (Houtz et al., 1992).

The enzyme: The most thoroughly studied eukaryotic N\(^{\text{\alpha}}\)-acetyltransferase activity is that of yeast Nats, which has been well summarized by Polevoda and Sherman (2003a); the table therein describes the subunits and substrate specificities of yeast NatA, NatB, and NatC types. The less studied NatD acetylates histones H4 and H2A (Song et al., 2003), and NatE awaits characterization of target substrates (Polevoda et al., 2008). None of the described activities would account for the modification of Pro3 of the large subunit of Rubisco.

Nat homologues in *Arabidopsis* organelles have been searched for by experts (Polevoda and Sherman, 2003b) and, though the cytosolic enzymes are discussed at length, the conclusion for organellar N\(^{\text{\alpha}}\)-acetyltransferases is that such enzymes are probably specific to the few acetylated proteins in the chloroplast. An *in silico* prediction of chloroplast localization of a putative acetyltransferase (Atg90740) was tested experimentally with transient expression of the green fluorescent protein (GFP) fusion (Fig. 3A); the fusion protein is obviously not localized in the chloroplast, as evident by the lack of yellow in the overlay between autofluorescence of chlorophyll and the GFP fusion.

Experimental evidence for cytosolic plant Nat homologues is present in the literature (Pesaresi et al., 2003), but enzyme localization had been determined solely on the basis of transient GFP fusion studies which lacked convincing controls. The potential still exists that this enzyme might function in the plastid.

Given the endosymbiotic origins of the chloroplast, a reasonable expectation is that plastidial N\(^{\text{\alpha}}\)-acyltransferase(s) would have sequence homology to the bacterial enzymes and might diverge from eukaryotic forms. Using that logic, sequence homology is most probable with RimL, RimJ, and RimL which N\(^{\text{\alpha}}\)-acetylate ribosomal proteins S18, S5, and L12, respectively (Yoshikawa et al., 1987; Tanaka et al., 1989). Together with the RimL structure (Vetting et al., 2005b), the structural co-ordinates for RimL (2CNT, 2CNS, and 2CNM; RCSB Protein Data Bank http://www.rcsb.org/pdb/home/home.do) should provide additional insight into the kinetic mechanism and substrate specificity of N\(^{\text{\alpha}}\)-acyltransferases generally regarding the stricter specificity of the bacterial acetyltransferase compared with that of Nats (Vetting et al., 2005b).

A more fruitful search for the acetyltransferase responsible for modifying the large subunit of Rubisco might be structure based and directed towards an odd class within the GNAT superfamily. The GNAT superfamily (GCN5-related N-acetyltransferase) is a large and sequence-divergent group of proteins which nevertheless are structurally comparable with recognized motifs of other acetyltransferases including yeast Nats (A–D; 100–120 amino acids) [reviewed first in Shaw et al.,...
acetylation. For instance, the modification increased or decreased a specific type of activity of the human hormone and neurotransmitter, respectively (as noted in Kendall et al., 1991; Wang et al., 1995), N\textsuperscript{3}-acetyltransferase abundance may well challenge traditional protein purification attempts. Given the non-essential nature of the enzymatic activity in yeast and the majority of phenotypes associated with the mutants hereto examined associated with the stages of high cell proliferation rates, a biochemical purification of N\textsuperscript{3}-acetyltransferase will be greatly facilitated by judicious choice of tissue as starting material, probably young, fast-growing, and greening leaves.

The functional significance: N-terminal modifications in general have been ascribed effects on folding, stability, sorting, degradation, and function (Kendall et al., 1990). Just as with the attempts to predict which proteins will functionally recognize a prolyl residue with a secondary amine group, the class of GNAT responsible might very well be different and yet probably still requires acetyl-CoA as a cofactor. The tools for conducting such structural searches and comparing known structures with entire structure databases are being developed [for example, MatAlign (Aung and Tan, 2006); ProtClass (Aung and Tan, 2005); 3D-BLAST (Tung et al., 2007; Yang and Tung, 2006); MAMMOTH (Ortiz et al., 2002); and CE (Shindyalov and Bourne, 1998)]. Given the high throughput crystallization efforts in Arabidopsis [(Jeon et al., 2005; Tyler et al., 2005); see http://www.ustructuralgenomics.org/ (confirmed 29 November 2007) for lists of publications including structural determination of ‘annotated-as-unknown’ Arabidopsis proteins], the identification of the acetyltransferase responsible for such an abundant chloroplast post-translational modification becomes a more definite and exciting possibility.

As with Rubisco large subunit methyltransferase (Rubisco LSMT) (Houtz et al., 1991; Wang et al., 1995), N\textsuperscript{3}-acetyltransferase abundance may well challenge traditional protein purification attempts. Given the non-essential nature of the enzymatic activity in yeast and the majority of phenotypes associated with the mutants hereto examined associated with the stages of high cell proliferation rates, a biochemical purification of N\textsuperscript{3}-acetyltransferase will be greatly facilitated by judicious choice of tissue as starting material, probably young, fast-growing, and greening leaves.

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A long history of proposals that the function of N\textsuperscript{3}-acetylation was protective against proteolytic degradation (Jornvall, 1975) includes that for Pro3 of the Rubisco LS (Houtz and Portis, 2003), namely against characterized leaf and chloroplastic iminopeptidases (Waters and Dalling, 1983; Liu and Jagendorf, 1986). The experimental study of such a role for N\textsuperscript{3}-acetylation is definitely complicated, and evidence, both for and against, has been presented (Driess et al., 1985).

The substoichiometric modification of L12 (acetylated L12 is known as L7) has been shown for both E. coli (Ramagopal and Subramanian, 1974) and Salmonella typhimurium (Abshire and Neidhardt, 1993) such that more L12 is present at early logarithmic growth compared with stationary phase and during shorter doubling times, respectively. Amounts of a modification may thus be a physiological state signal to the cell (Kaczanowska and Ryden-Aulin, 2007). Given that the area just below the L7/L12 stalk in the ribosome is a region of high protein concentration and a binding site for many translation factors (Kaczanowska and Ryden-Aulin, 2007), it is tempting to suggest that the modification state of L7/L12 may have a role to play in the fine-tuning of the recruitment of said translation factors. Indeed, another bacterial protein, the Mycobacterium tuberculosis ESAT-6 (6 kDa early secreted antigenic target) has recently been characterized as having multiple forms, including N\textsuperscript{3}-acetylation; CFP10 (10 kDa culture filtrate protein) recognizes ESAT-6 only in a non-acetylated form (Okkels et al., 2004). The functional significance of this particular interaction is unknown.

Although N\textsuperscript{3}-acetylation has been presumed to be non-reversible (Glozak et al., 2005), the same was true of methylation until identification of at least two classes of demethylases which have AOL/SWIRM/TOWER and Jumanjii domains (Anand and Marmorstein, 2007). Curiously, the targets of class II histone deacetylases (HDACs) are unknown (Gloza et al., 2005).

LS methylation

Methylation at Lys14 in the LS is a species-specific modification and is not predictable except through direct determination (Houtz et al., 1989, 1992). For many years, the role of Lys14 methylation has remained unknown despite the fact that the enzyme responsible, Rubisco LSMT, has been extensively studied and characterized from pea (Pisum sativum) (Wang et al., 1995; Klein and Houtz, 1995; Zheng et al., 1998). Rubisco LSMT is a SET domain protein methyltransferase, structurally similar to many histone methyltransferases which have received intense scrutiny as determinants in the regulation of epigenetic gene expression (Trievel, 2004; Lee et al., 2005; Shilatifard, 2006). The intense interest and voluminous research on histone methyltransferases, as well as the functional consequences of histone methylation, have provided important avenues of research for Rubisco.
LSMT, with many similarities as well as important differences. Rubisco LSMT utilizes a processive reaction mechanism to catalyse exclusively trimethylation of Lys14 without dissociation from Rubisco (Dirk et al., 2007). Thus, mono- and dimethyl forms of Lys14 are not observed, whereas in the N-terminal region of histones all three forms of methylated lysyl residues can be found. However, similar to histone methyltransferases, recent evidence has demonstrated that SET domain protein methyltransferases are capable of recognizing and methylating multiple protein substrates (Chuikov et al., 2004; Kouskouti et al., 2004; Zhang et al., 2005; Huang et al., 2006). Since Rubisco LSMT is strictly chloroplast localized (Fig. 3B), alternative substrates are likely to be components of photosynthetic pathways. Two approaches have been used to investigate the substrate specificity and alternative protein substrates for Rubisco LSMT. The first approach examined flexibility in the polypeptide substrate specificity of Rubisco LSMT using an alternative substrate consisting of a fusion protein between the C-terminus of the N-terminal region of the Rubisco LS and the N-terminus of human carbonic anhydrase II (Magnani et al., 2007). Site-directed mutagenesis of the sequence and residues flanking the Lys14 methylation site was used to map the polypeptide specificity determinants for Rubisco LSMT. A consensus sequence was identified and, through a bioinformatics search, several other chloroplast-localized proteins were identified as potential protein substrates for Rubisco LSMT. One of these, γ-tocopherol methyltransferase (TMT, EC 2.1.1.95), was confirmed as a substrate for Rubisco LSMT through in vitro studies. However, in vitro studies cannot unequivocally predict a protein’s in vivo methylation status given protein folding and membrane localization constraints. Therefore, a second approach for investigating alternative protein substrates for Rubisco LSMT was developed, which also serves as a reliable method for confirming predicted polypeptide substrates from the aforementioned substrate specificity studies. This approach relies on the creation of open methylation sites in proteins which are normally methylated by Rubisco LSMT using RNA interference (RNAi)-mediated knockdown of Rubisco LSMT in tobacco plants. Incubation of chloroplast extracts from Rubisco LSMT knockdown plants with cloned pea Rubisco LSMT reveals a number of polypeptide substrates in addition to the LS of Rubisco. Identification of these polypeptide substrates is ongoing, but one already confirmed is fructose-1,6-bisphosphate aldolase (EC 4.1.2.13). In this case, as well as for TMT, enzyme activity seems to be down-regulated in the absence of methylation. These results have also prompted a re-examination of the effects of methylation on Rubisco. Earlier studies, which did not reveal any changes in activity, relied on the introduction of Lys14 methylation into a non-methylated Rubisco species such as spinach Rubisco. Thus, the functional consequences of Lys14 methylation may only be manifested by methylated forms of Rubisco where methylation has been removed. How methylation could affect enzyme activity is open to speculation, but the functional consequences of histone methylation have been well described. Site-specific methylation of lysyl residues in histones creates recognition sites where proteins with binding domains specific for methylated lysyl residues are recruited. Currently, there are a number of binding domains for methylated lysyl residues found in proteins including Chromo (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Flanagan et al., 2005; Wysocka et al., 2005; Klymenko et al., 2006; Pray-Grant et al., 2005), JmJ C (Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006), Tudor (Botuyan et al., 2006; Huang et al., 2006b; Kim et al., 2006), MBT (Kim et al., 2006; Trojer et al., 2007), and PHD (Li et al., 2006; Martin et al., 2006; Shi et al., 2006, 2007; Wysocka et al., 2006) domains. Similar to the substrate specificity of methyltransferases, proteins containing these domains, sometimes in unique context with other domains, have been shown to recognize a given lysyl residue in a specific state of methylation (Maurer-Stroh et al., 2003; Daniel et al., 2005; Anand and Marmorstein, 2007; Cheng and Zhang, 2007). For instance, the chromodomain of HP1 binds to monomethylated Lys9 in histone H3 (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001), and yet the chromodomain of CDY1 interacts with the di- or trimethylated version of the same lysine in the same histone (Kim et al., 2006). The possibility of chloroplast-localized proteins with binding domains specific for methylated lysyl residues remains to be investigated, although, as mentioned earlier, bioinformatic approaches that identify proteins targeted to chloroplasts have to be experimentally verified. Nevertheless, Rubisco, as well as many other photosynthetic enzymes, has been reported to interact with different proteins (Portis et al., 1986; Anderson and Carol, 2004) as well as to form supercomplexes (Suss et al., 1995; Long et al., 2007). Thus, it is possible that these interactions are a consequence of, or potentially regulated by, the presence or absence of methylated lysyl residues at strategic locations within the proteins. It is worth noting that all of the existing studies of Rubisco LSMT have utilized the pea form of the enzyme, but homologues are interestingly found in all plant species, even those without Lys14 methylation. An earlier report from the authors’ laboratory has indicated that, in some species, alternatively spliced forms of Rubisco LSMT may be responsible for methylation of Met1 in the SS (see below), but these studies have not been confirmed by others. At least one other Rubisco LSMT, from Arabidopsis thaliana, has been directly characterized as possessing LSMT activity, as indicated in Fig. 3C, and has been correctly annotated with a rare GC intron (see http://www.arabidopsis.org At1g14030 locus information).
Other LS modifications
At least two other amino acid modifications have been described in Rubisco from C. reinhardtii, but these have not been found in vascular plant forms of Rubisco. These include a methylated cysteine residue and two 4-hydroxyproline residues for which there are no known functions.

SS transit peptide removal and methylation
Post-translational processing of the SS involves cleavage of an ~5 kDa polypeptide from the N-terminus of the precursor form of the polypeptide (Chua and Schmidt, 1978) (Fig. 1B). All indications are that this reaction is catalysed by a stromal processing peptidase (SPP) which has been extensively characterized and has activity towards a number of chloroplast-targeted proteins (VanderVere et al., 1995; Richter and Lamppa, 1998, 2003). Following transit peptide removal, the exposed N-terminal methionine residue is methylated on the α-amino group and this modification is found in all vascular plant Rubiscos examined (Grimm et al., 1997; Houtz and Portis, 2003). Studies of SS processing where the SS gene has been relocated to the chloroplast genome suggest that the methyltransferase responsible for Met1 methylation may be localized to the protein import apparatus (Whitney and Andrews, 2001). As mentioned earlier, at least one study has suggested that the SS methyltransferase is related to Rubisco LSMT, with alterations in activity and specificity as a consequence of alternative RNA splicing (Ying et al., 1999). However, there is at least one other example of N-terminal targeting sequence cleavage which is followed by N-terminal methylation. Preprotein peptides, most notably PilD, cleave leader peptides or pre-peptides from prepilins and are bifunctional enzymes with both peptidase and N²-acetyltransferase activities located at two distinct catalytic sites (Nunn and Lory, 1991; Strom et al., 1991, 1993; Strom and Lory, 1992). Type IV prepilins are processed in this way for secretion and assembly into a surface organelle, the pilus, used for multiple purposes including DNA uptake, motility, and attachment (Strom et al., 1994; Lory and Strom, 1997; Filloux et al., 1998). Leader peptide removal is essential for subsequent pilus assembly, but methylation of the N-terminal phenylalanine residue is not and its function remains unknown (Pepe and Lory, 1998). There are no identifiable orthologues of PilD in databases of Arabidopsis proteins (data not shown) and, if similar plant enzymes exist, more sophisticated methods for comparisons may be necessary for their identification.

Conclusions
The relatively large number of co- and post-translational modifications in the LS and SS of Rubisco leading up to the final mature protein forms encompass a number of enzymatic processes that despite similarity with other systems have unique features that make them fertile ground for scientific research. The widespread occurrence of MAPs, N²-acetyltransferases, demethylases, deacetylases, and aminopeptidases suggests that the chloroplast-localized orthologues may have significant structural and/or sequence similarities. More importantly, many of the processes catalysed by these enzymes are essential and thus may play important roles in chloroplast biology beyond their importance in the processing of Rubisco subunits. Given the conservation of LS and SS sequences surrounding the co- and post-translational processing sites, one would expect the enzymes involved to be conserved across a wide range of plant species and amenable to in silico analyses with the number of plant genomes available. Hopefully, the unanswered questions provided here will stimulate others to examine the structural modifications in Rubisco at the genetic and biochemical levels and help complete our understanding about functional and mechanistic aspects of co- and post-translational modifications in Rubisco.

Acknowledgements
Our thanks for technical efforts for confocal imaging go to Dr Randy D Dinkins, Research Plant Molecular Geneticist at USDA/ARS in the Forage Animal Production Research Unit. We also acknowledge Dr Brent W Meier for contributing the demonstration of the activity of the Arabidopsis Rubisco LSMT. This work was supported by the Department of Energy grant (#DE-FG02-92ER20075) to RLH.

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