Protein Methylation in Pea Chloroplasts

Kevin J. Niemi*, Julius Adler, and Bruce R. Selman

Department of Biochemistry (K.J.N., J.A., B.R.S.) and Department of Genetics (J.A.), University of Wisconsin-Madison, Madison, Wisconsin 53706

ABSTRACT

The methylation of chloroplast proteins has been investigated by incubating intact pea (Pisum sativum) chloroplasts with [3H]-methyl-S-adenosylmethionine. Incubation in the light increases the amount of methylation in both the thylakoid and stromal fractions. Numerous thylakoid proteins serve as substrates for the methyltransfer reactions. Three of these thylakoid proteins are methylated to a significantly greater extent in the light than in the dark. One is a polypeptide with a molecular mass of 135 kDa, a second has an M, of 48 kDa, and the third has a molecular mass of less than 10 kDa. The primary stromal polypeptide methylated is the large subunit of rubisco, while a second type is base labile. The base-stable linkage is indicative of N-methylation of amino acid residues while base-lability is suggestive of carboxylation of amino acid residues. Labeling in the light increases the percentage of methylation that is base labile in the thylakoid fraction while no difference is observed in the amount of base-labile methylations in light-labeled and dark-labeled stromal proteins. Also suggestive of carboxymethylation is the detection of volatile [3H]methyl radioactivity which increases during the labeling period and is greater in chloroplasts labeled in the light as opposed to being labeled in the dark; this implies in vivo turnover of the [3H]methyl group.

Many types of posttranslational modifications of proteins occur in both prokaryotic and eukaryotic organisms. Examples include phosphorylation, glycosylation, and methylation.

Protein methylation in prokaryotes is exemplified by Escherichia coli and Halobacterium halobium. In E. coli chemotaxis, attractant or repellent chemicals interact with cytoplasmic-membrane receptor proteins that signal to the flagella to create the desired movement of the bacterium either toward or away from the chemicals; then this signal is terminated by glutamate-carboxyl methyltransfer reactions in these receptor proteins in the case of attractants and demethylation in the case of repellents (7, 14, 15, 22, 29, 31). The methylating agent is AdoMet (23), and the demethylating product is methanol (14, 31), which is excreted (28). In H. halobium phototaxis, cytoplasmic-membrane proteins are methylated in attractant visible light and demethylated in repellent ultraviolet light (1, 4, 21, 24).

Protein methylation in plants has not been thoroughly characterized. Gupta et al. (11) reported a protein methylase involved in the methylation of arginine residues in histone proteins of wheat germ. Black et al. (5) described methylation of proteins in freshly broken spinach chloroplasts and concluded that both light-dependent and light-independent methylation of chloroplast proteins occurs. These authors concluded that at high concentrations of AdoMet methylation appears to be nonspecific and that possibly it has a similar function to that found in the human erythrocyte (2), namely to serve as a repair mechanism for racemized aspartic acid residues. At low concentrations of AdoMet, however, they concluded that the methyl-transfer reactions appear to be more specific with respect to their substrates. The only identified substrates for posttranslational methylation in chloroplasts are the large and small subunits of RuBPCase as described by Black et al. (5) and Houtz et al. (12). The role of specific protein methylation in plants is not known.

This project was developed with the endosymbiotic theory in mind. This theory proposes that eukaryotic organelles are derived from ancient prokaryotes (20). Modern prokaryotes such as those described above utilize protein glutamate-carboxyl methylation in their chemotactic and phototactic responses. Our investigation was initiated to examine whether similar methylation reactions occur in the chloroplast. We wanted to determine the extent of protein methylation in pea chloroplasts and to find out whether protein methylation is involved in their light-responsive events.

MATERIALS AND METHODS

Plant Material

Pea (Pisum sativum) plants were grown under a 12 h photoperiod in a 1:1, vermiculite:perlite mixture in a temperature-controlled, 25°C/18°C day/night, growth chamber. Light levels were approximately 300 to 400 µE m⁻² s⁻¹ at plant height.

Chloroplast Isolation

Chloroplasts were isolated by the following modified procedure of Bartlett et al. (3). Pea leaves 14 to 24 d old were ground in ice-cold grinding buffer consisting of 50 mM Hepes/KOH (pH 7.5), 330 mM sorbitol, 2 mM K-EDTA, 1 mM MgCl₂, 1 mM MnCl₂, and 5 mM isoascorbate for 5 to 10 s in

---

1 Supported by National Science Foundation grant BNS-8804849 to J. A. and National Institutes of Health grant GM 31384 to B. R. S.

2 Abbreviations: AdoMet, S-adenosylmethionine; RuBPCase, ribulose bisphosphate carboxylase/oxygenase; SAH, S-adenosylhomocysteine.
a Waring blender. The slurry was filtered through four layers of Miracloth. Chloroplasts were pelleted by centrifugation for 3 min at 3,000g. The pellet was suspended in 1 to 2 mL of grinding buffer. This suspension was placed on a preformed (40 min at 35,000g) linear, 15 to 80% Percoll gradient and centrifuged for 10 min at 8,000g, and the rotor was allowed to stop without braking. The lower band in the gradient was removed by pipetting and diluted with three volumes of ice-cold resuspension buffer consisting of 66 mM Tricine/KOH (pH 8.3), 200 mM KCl, and 6.6 mM MgCl₂. The suspension was centrifuged for 6 min at 1,500g and again allowed to stop without braking. The pellet was dispersed in resuspension buffer. Isolation procedures were performed at 4°C.

The Chl concentration was determined spectrophotometrically by suspension in 80% acetone using the extinction coefficients of Lichtenhaler (16). Chloroplast intactness was assessed by ferricyanide reduction and phase contrast microscopy.

**Labeling Conditions**

Chloroplasts, at 0.3 to 1.0 mg Chl/mL resuspension buffer, were preincubated in either the light or dark (foil-wrapped tubes) for 15 min at 25°C in 15 mL Pyrex tubes in a clear-bottom temperature-controlled circulating bath. The tubes were 24 cm above a General Electric 400 W high-intensity discharge lamp. After the preincubation period, 0.3 μM [3H]-methyl[5]-adenosyl-L-methionine, (55–85 Ci/mMol, Dupont) was added for 5 to 30 min. The contents were then transferred to 1.5 mL polypropylene microcentrifuge tubes and fractionated as described below.

**Fractionation**

The radioactive chloroplasts were sedimented in a microfuge and resuspended in 10 mM NaPPi/HCl (pH 6.5), and placed on ice for 15 min to rupture the chloroplasts. Thylakoid membranes were pelleted by centrifugation for 5 min in a microfuge at 10,000g, resuspended in 10 mM NaPPi/HCl (pH 6.5), and repelleted. The two supernatants were combined for the soluble, stromal fraction. Thylakoid membranes were washed three more times with 10 mM NaPPi/HCl (pH 6.5), with pelleting each time for 10 min at 10,000g. The final resuspensions of thylakoid membranes were in 0.2 mM NaPPi/HCl (pH 6.5). Then the thylakoid and the stromal fractions were both precipitated with either 5 volumes of -20°C acetone or with the addition of TCA to 5%, put on ice for 30 min, and then pelleted for 10 min at 10,000g. To further reduce the unincorporated background radiation, the pellets were resuspended in 0.2 mL of 100 mM NaPPi/HCl (pH 6.5) and precipitated with either acetone or TCA as described above. The pellets were solubilized in 10% SDS (w/v). Aliquots were counted for radioactivity in ReadySafe scintillation fluid in a Packard Tri-Carb 460C. Protein was determined by the Lowry method as modified by Markwell with use of bovine serum albumin as a standard (17).

**Inhibitor Studies**

The blockage of photosynthetic electron transport was accomplished by including 5 μM DCMU during the labeling reactions.

The inhibition of methylation by SAH was assessed by including varying concentrations of SAH from 5 nM to 5 mM during incubation of chloroplasts with the methyl-labeled AdoMet. Following incubation the samples were treated as described above.

**Volatile Radioactivity Produced by Alkaline Hydrolysis with Heating**

In a microdistillation technique (26), base was added (0.1 mL of 1 M Na₂CO₃) to 13 × 100 mm test tubes that contained an aliquot of the labeled sample. H₂O replaced the base in control tubes. The tubes were capped with serum caps and heated to 80°C. The volatile radioactive components were vented via a syringe needle and tygon tubing into a scintillation vial containing 10 mL of ReadySafe scintillation fluid. After 30 min and 45 min, 1 mL cold methanol chase was added with a syringe to fully purge the volatile radioactivity from the test tubes into the scintillation fluid. After 60 min the scintillation vials were counted for radioactivity. Counts were corrected for methanol quenching (using the sample channels ratio method) and the efficiency of the microdistillation technique. The efficiency of microdistillation (69%) was determined using 14C-labeled methanol.

**Volatile Radioactivity Produced Without Alkaline Hydrolysis and Without Heating**

The Fettes et al. (10) modification of the vapor equilibration method of Chelsky et al. (8) was employed. Aliquots of the labeling mixture were removed at time points, TCA precipitated (final concentration 5%), and centrifuged for 10 min at 16,000g. The supernatant was transferred to a polypropylene microcentrifuge tube, 0.05 mL of 20% (w/v) phosphotungstic acid and 10 μmol unlabeled AdoMet were added, the tube was closed, and the mixture was incubated overnight at 4°C to precipitate AdoMet, which was then removed by centrifugation for 60 min at 16,000g at 4°C. The supernatant was assayed for volatile [3H]methyl radioactivity by putting it in a polypropylene microcentrifuge tube and placing the open tube into a scintillation vial containing 10 mL of ReadySafe scintillation fluid. The scintillation vial was sealed and set aside at room temperature for 4 to 48 h. The scintillation vials were then counted (without removing the microcentrifuge tube from within) in a liquid scintillation counter. The counts detected are indicative of volatile [3H]methyl groups.

**SDS-PAGE/Fluorography**

Fractions containing equal amounts of radioactivity were analyzed on polyacrylamide gels (7.5 to 15% linear gradient Laemmli gels with a 4% stacking gel). Fluorography was performed using DMSO and PPO. The gels, sealed between cellophane sheets and dried, were exposed at -80°C to preflashed Kodak X-Omat AR film for a number of weeks. After the exposure period, the gels were rehydrated in 10% acetic acid, the cellophane was removed, and the PPO was removed from the gel by soaking in DMSO for at least 24 h. The gels were then stained with Coomassie brilliant blue...
in methanol:H₂O:acetic acid (4.5:4.5:1, v/v) and destained with H₂O:methanol:acetic acid:glycerol (5.15:4.0:7.5:0.1, v/v).

RESULTS

Light-Enhanced Methylation

TCA-insoluble compounds became methylated when intact pea chloroplasts were incubated with [³H-methyl]S-adenosylmethionine (Table I). Stromal-fraction compounds were much more heavily methylated than thylakoid-fraction compounds. The methylation of both fractions increased in the light albeit at different rates. For example, after a 5 min exposure to light, the increase in the incorporation of methyl label into the stromal fraction amounted to approximately 25% whereas the thylakoid fraction had only slightly higher amounts of label than when labeled under dark conditions. After an additional 10 min of labeling, the methylation of the stromal fraction did not increase. In contrast, the additional 10 min exposure to light further increased the difference between the light- and dark-labeled thylakoid fraction (light, 142% increase; dark, 54% increase). The level of incorporation of label into the thylakoid fraction after 15 min in the light was about 70% above the dark-labeled amount. Black et al. (5) reported similar light effects on the methylation level of proteins in broken spinach chloroplasts.

Labeled Polypeptides

Exhaustive pronase treatment of these TCA-precipitable products renders all radioactivity TCA-soluble and not visible on a fluorograph (data not shown), so the observed radioactivity is indeed in protein.

Figure 1 shows the polypeptides obtained from thylakoid and stromal fractions. The Coomassie-stained polypeptide profile of the thylakoid and stromal fractions is shown in panel A of Figure 1. A comparison of these polypeptide profiles (panel A) and the fluorographed (radioactive) methylation profiles (panel B) clearly shows that the polypeptides are not methylated in proportion to their abundance. There are numerous examples of polypeptides that are undermethylated relative to their abundance, e.g. thylakoid polypeptide with a molecular mass of 25 kD, the most prevalent Coomassie-stained polypeptide in lane 1 of A gave no evidence of methylation in the corresponding fluorogram, lane 1 of B. There are also examples of polypeptides not readily evident in the Coomassie-stained lanes of panel A but very evident in the fluorogram B, e.g. the polypeptide with Mᵣ around 45 kD in the thylakoid-fraction lane 1. Similar examples are also evident in the stromal fraction. It is clear that the methylation level is not proportional to the amount of a polypeptide present in the chloroplast.

Of interest in the thylakoid fraction are several polypeptides that are methylated more in the light than in the dark (Fig. 1, panel B, closed arrowheads; lane 1, light versus lane 3, dark). The molecular masses of these polypeptides are about 64, 48, and <10 kD. The inclusion of DCMU during the incubation of chloroplasts with AdoMet in the light results in decreased methylation of these three polypeptides (Fig. 1, panel B, lanes 5 and 7).

The bulk of the label in the stromal fraction appears in a polypeptide with an apparent molecular mass of 50 to 55 kD (Fig. 1, panel B, lanes 2 and 4). This polypeptide is methylated in both light- and dark-labeled conditions. This methylated polypeptide is most likely the large subunit of RuBPCase. Houtz et al. (12) reported the methylation of specific lysine residues in the N-terminal region of the large subunit in several other species. The stromal fraction also contains a polypeptide of molecular mass about 24 kD that is methylated more in the light than in the dark (Fig. 1, panel B, open arrowhead; lane 2, light versus lane 4, dark). The identity of this polypeptide is not known. Several other stromal polypeptides are also methylated.

The methylation reactions are enzymatic in that the inclusion of SAH, an inhibitor of methyltransferase enzymes (6), during the labeling incubations drastically reduced the amount of methylation. Micromolar concentrations of SAH were sufficient to cause a 50% reduction and millimolar concentrations totally inhibited transfer from methyl-labeled AdoMet to chloroplast protein (data not shown).

Base-Labile versus Base-Stable Methylation

Methyl groups transferred to chloroplast proteins from methyl-labeled AdoMet were found in both base-labile and base-stable linkages. As shown in Table II, about 5 to 20% of the methyl groups incorporated into chloroplast protein were base labile. The remainder of the label was not hydrolyzed under these basic conditions. In the thylakoid fraction light-labeled samples had a higher proportion of base-labile linkages than dark-labeled samples. In contrast the light conditions during labeling had no effect on the percentage of base-labile and base-stable linkages in the stromal fraction.

Volatile Radioactivity Production

Figure 2 presents data indicating the production of a volatile [³H]methyl radioactive compound during the labeling period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Label Transferred</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stromal</td>
<td>Thylakoid</td>
</tr>
<tr>
<td></td>
<td>pmol mg⁻¹ protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Minute labeling period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>30.7 ± 1.96</td>
<td>0.301 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>24.8 ± 1.54</td>
<td>0.280 ± 0.038</td>
<td></td>
</tr>
<tr>
<td>15 Minute labeling period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>28.6 ± 5.29</td>
<td>0.730 ± 0.098</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>22.2 ± 1.87</td>
<td>0.431 ± 0.113</td>
<td></td>
</tr>
</tbody>
</table>
The amount of volatile radioactivity increases over the duration of the labeling period when the chloroplasts are labeled in the light. There is also a slight lag in the period of time when the [3H]methyl volatile products are detected; volatile [3H]methyl is not detected at ten minutes but rises steadily after 20 and 30 min of labeling. The amount of [3H]methyl volatile radioactivity produced by chloroplasts incubated in the dark is only slightly above the background level of volatile radioactivity.

**DISCUSSION**

Several of our findings indicate a light-responsive role for protein methylation in the chloroplast. First, several polypeptides are methylated to a greater degree in the light. Second, there is an increase in the amount of base-labile linkages in thylakoid protein when chloroplasts are labeled in the light. Third, chloroplasts labeled in the light show an increasing amount of volatile [3H]methyl radioactivity. This volatile

**Table II.** Volatile [3H]Methyl Radioactivity Produced by Alkaline Hydrolysis with Heating of Transferred [3H]Methyl Label in Chloroplast Fractions

<table>
<thead>
<tr>
<th>Labeling Condition</th>
<th>Base-Labile Linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stromal</td>
</tr>
<tr>
<td>Light</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>Dark</td>
<td>17.8 ± 1.5</td>
</tr>
</tbody>
</table>

Figure 1. Methylation profile of pea chloroplast polypeptides. Intact pea chloroplasts were incubated with [3H-methyl]AdoMet for 15 min, fractionated, separated by a 7.5 to 15% linear gradient SDS-PAGE gel, and fluorographed as described in "Materials and Methods." A, Coomassie-stained polypeptide profile; B, fluorograph of [3H]methylated polypeptides. Fraction: t, thylakoid; s, stromal. Light: +, labeled in light; −, labeled in dark. DCMU: +, inclusion of 5 μM DCMU during labeling; −, no DCMU added. Arrowheads denote position of polypeptides that are methylated to a greater degree in light; ( ), thylakoid fraction; ( ), stromal fraction. Equal cpm were loaded in each lane. Lanes containing mol wt standards are indicated by diamonds (∗). The apparent mol wt of standards is shown at the left. The positions of the standards on fluorogram were identified with a marking pen. Standards were from a Bio-Rad prestained SDS-PAGE standards kit (130 kD, phosphorylase b; 75 kD, BSA; 50 kD, ovalbumin; 39 kD, carbonic anhydrase; 27 kD, soybean trypsin inhibitor; 17 kD, lysozyme).
radioactivity produced by pea chloroplasts may well be methanol, and this would be indicative of in situ turnover of carboxymethyltransferases.

At least five amino-acid residues of proteins have been reported to be acceptors of the methyl group from AdoMet. The methyl esters of glutamate and also aspartate are base labile (13, 27). Hydrolysis of aspartate and glutamate methyltransferases produces methanol. Base-stable methyl-accepting sites are arginine (30), histidine (18), and lysine (12, 19). We found both base-labile and base-stable linkages: under our labeling conditions 20% or less of the incorporated methyl groups are base labile. Black et al. (5) reported that 60 to 70% of the label incorporated into spinach chloroplast proteins was base labile. This discrepancy in the amounts of base-labile and base-stable linkages in spinach and pea chloroplast proteins may result from the lack of methylated lysine residues in the N-terminal region of spinach RuBPCase (12), from the different methods used to measure the amount of base-labile and base-stable linkages, or from the different labeling and washing methods used.

At least two classes of protein carboxyl methyltransferase enzymes have been characterized (9). The carboxymethylation of glutamate residues in prokaryotes involves class I carboxymethyltransferase enzymes. These have to date been found only in prokaryotes and are involved in their tactic responses. Class II protein carboxymethyltransferase enzymes are thought to identify D-aspartyl residues in protein for repair to the L-form in erythrocytes (9) and are ubiquitous in both prokaryotes and eukaryotes. The predominant class of methyltransferase activity in broken spinach chloroplasts was reported by Black et al. (5) to be class II carboxymethyltransferase activity. The methylation pattern in chloroplast polypeptides which indicates a light/dark difference could arise from either carboxyl- or N-methylations. This is under investigation.

The chemotactic system of E. coli and the phototactic system of H. halobium both involve methylation of membrane proteins (see Introduction). It has been demonstrated that the turnover of the methyl group on esters of glutamate residues via a methyl esterase (25) produces methanol (14, 31). This methyl group turnover is involved in the adaptation response of the organism to the external stimulus. Some properties of the methyltransferases involved in the tactic response in prokaryotes are base-labile methylation of glutamate residues in protein and production of volatile methanol by enzymatic hydrolysis of the methyltransferases, which is influenced by external stimuli. We have reason to believe that chloroplast methylation fits several of these criteria. Our volatile [3H]methyl detection may arise from either aspartate- or glutamate-ester hydrolysis, or both. However, the detection of greater amounts of volatile [3H]methyl radioactivity due to the labeling condition, i.e. light versus dark, and the observation of light-dependent protein methylations makes glutamate carboxymethylation reasonable to suspect.

In conclusion, we present evidence for the post-translational methylation of many chloroplast polypeptides. Several of the polypeptides are methylated to a greater extent in the light than in the dark. More numerous are polypeptides methylated equally in both light and dark. We present data supporting two distinct types of methyl-linkages in the proteins of intact pea chloroplasts. One methyl-linkage produces volatile radioactivity and is base labile, properties similar to carboxymethylated proteins involved in the chemotactic and phototactic responses of prokaryotes. The other methyl-linkage is base stable and apparently involves N-methylations of amino acid residues.

LITERATURE CITED

10. Fetters HA, Kellerer J, Duerr JA (1985) Protein-carboxyl...