Registry No. ISO, 51-31-0; adenylate cyclase, 9012-42-4.

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Occurrence of a Methylated Protein in Chloroplast Ribosomes†

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ABSTRACT: A ribosomal protein of spinach chloroplast, previously shown to be immunologically homologous to *Escherichia coli* ribosomal protein L2, was purified by using gel filtration, ion-exchange chromatography, and reverse-phase high-performance liquid chromatography separation. Analysis of the N-terminal amino acid sequence of the purified protein and of the fragments obtained by its cyanogen bromide cleavage shows that *N*-methylalanine is the N-terminal residue of this protein. Thus, methyl modification of proteins, whose occurrence in *E. coli* ribosomes is established, is present in chloroplast ribosomes as well. This and the additional significance of the amino acid sequence data reported in this paper are discussed.

The most common modification of both ribosomal protein (r-protein)[1] and rRNA of ribosomes is methylation (Wittmann, 1982; Fellner, 1974). *Escherichia coli* ribosomes contain six methylated proteins, five of them in the large subunit (L3, L11, L12, L16, and L33) and one (S11) in the small subunit (Wittmann-Liebold, 1980). Genetic studies have shown that methyl modifications in *E. coli* are catalyzed by specific methylases (Colson et al., 1979; Isono, 1980). However, as far as is known, the methyl (and other) modifications in themselves are not essential for the basic translational function of the bacterial ribosome (Isono, 1980). It would therefore be important to know, for evaluating their biochemical significance, whether r-protein modifications have been deleted out in ribosomes from organisms that have diverged from bacteria into alternative evolutionary pathways in the very distant past.

Chloroplast ribosomes display the prokaryotic ribosome motif in many of their structural and functional properties even though they exist in the eukaryotic cell and have many of their protein components encoded in the nuclear DNA (Bogorad et al., 1977; Ledoigt & Freyssinet, 1982; Subramanian, 1985). The origin of chloroplast evolution is placed between 500 ×

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[1] Abbreviations: r-protein, ribosomal protein; rRNA, ribosomal RNA; NM-Ala and *N*-methylalanine, α-*N*-monomethylalanine; SDS, sodium dodecyl sulfate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene thiohydantoin; HPLC, high-performance liquid chromatography.

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10^6 and 1000×10^6 years ago (Hoober, 1984).

In the present paper, we describe the purification of a spinach chloroplast r-protein that was previously shown to cross-react specifically with antisera against *E. coli* r-protein L2 (Bartsch, 1985). Structural analysis shows that the N-terminal residue of this protein is *N*-methylalanine, thus providing the first evidence (as far as we know) for protein methylation in the chloroplast ribosome.

Materials and Methods

Preparation of Chloroplast, Chloroplast Ribosome, and r-Proteins. Chloroplasts were prepared from fresh spinach (*Spinacia oleracea*, cultivar Alwaro) leaves, and ribosomes were isolated from them, as described previously (Bartsch et al., 1982). About 400 mg (6400 A_{260} units) of chloroplast ribosomes was on the average obtained from 10 kg of leaves. The ribosomes were extracted with ethanol/1 M NH_4Cl to remove r-protein L12 which was earlier purified and sequenced (Bartsch et al., 1982). The residual ribosome was stirred with 67% acetic acid/0.05 M magnesium acetate at 0 °C for 1 h, and the soluble protein extract obtained by centrifugation (Hardy et al., 1969) was lyophilized after extensive dialysis against 5% acetic acid.

r-Protein Purification. This was done in three sequential steps: (1) size fractionation by gel filtration through a column of Sephadex G-100 superfine; (2) ion-exchange chromatography of most of the pooled fractions obtained from gel filtration; and finally (3) reverse-phase HPLC purification of the partially pure fractions from the earlier steps.

Lyophilized r-proteins (~1 g) obtained from 3 g of chloroplast ribosomes were dissolved in 6 M urea/5% acetic acid and applied on a 4 cm × 80 cm column of Sephadex G-100 superfine (Pharmacia) equilibrated with 6 M urea/5% acetic acid and eluted with the same solution. The collected fractions were analyzed by SDS gel and two-dimensional gel electrophoresis (Laemmli, 1970; Subramanian, 1974) and were pooled on the basis of this information.

The pooled proteins from the gel filtration column were chromatographed on columns (1 × 20 cm) of carboxymethyl-Sephadex C-25, carboxymethyl-Sepharose CL-6B (both from Pharmacia), and/or phosphocellulose (Serva) equilibrated with 6 M urea, 0.01 M sodium acetate, pH 5.6, and 0.006 M 2-mercaptoethanol. After the columns were washed with the equilibration buffer, the proteins were eluted with 500 mL of a 0–0.5 M NaCl gradient in the same buffer. The collected fractions were analyzed by electrophoresis as before and were pooled. In most cases, the pools contained two to six components, but in several cases, a single component was obtained. The pools were concentrated by Diaflo filtration (YM5 membrane, Amicon) and were stored at −80 °C. Before application on HPLC columns, they were desalted by extensive dialysis against 10% acetic acid at 4 °C and were dried in a Speed-vac concentrator (Savant).

Reverse-Phase HPLC. For the HPLC purification of L2 and its CNBr-cleaved fragments, a Du Pont instrument (Model 850) equipped with a variable wavelength detector (Du Pont Model 852) and an automatic sampler (WISP 710A, Waters Associates) was used. Reversed-phase separation was performed on laboratory-packed Vydac TP-RP (C18, particle size 5 μm, pore size 300 Å) columns (250 × 4.6 mm). Proteins or CNBr peptides were eluted with a gradient, from 0.1% trifluoroacetic acid in H_2O to 0.1% trifluoroacetic acid in 2-propanol, at a flow rate of 0.5 mL/min.

CNBr Cleavage of Protein L2. Ten nanomoles of the purified protein (M_r 30K; Bartsch, 1985), dissolved in 200 μL of 70% formic acid, was treated with a 10-fold molar excess of CNBr at room temperature for 24 h. The sample was dried in vacuo and was purified on the HPLC column.

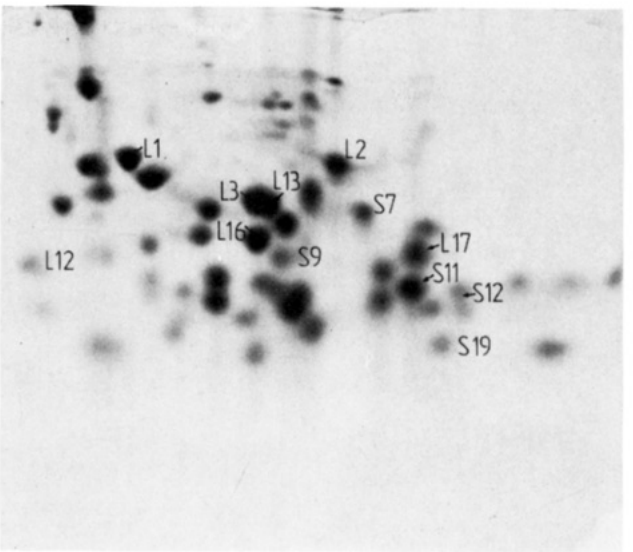

FIGURE 1: Spinach chloroplast ribosomal proteins separated by two-dimensional acrylamide gel electrophoresis. The positions of L2 and 11 other r-proteins that each cross-react with antisera against the corresponding purified *E. coli* r-protein (Bartsch, 1985) are shown. Electrophoresis (Subramanian, 1974) from left to right was in the presence of 8 M urea (pH 5.0) and from top to bottom was in the presence of sodium dodecyl sulfate (pH 6.8).

Amino Acid Analysis. The proteins were hydrolyzed in 100 μL of twice-distilled 5.7 N HCl containing 0.02% 2-mercaptoethanol at 110 °C for 20 h. Portions of the hydrolysate were analyzed on a Durrum D-500 analyzer using postcolumn ninhydrin derivatization or on a Waters HPLC system using precolumn *o*-phthaldialdehyde derivatization techniques (Ashman & Bosserhoff, 1985).

Microsequencing. Sequencing of protein and CNBr peptides was performed manually by the 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate/phenyl isothiocyanate double coupling method (Chang et al., 1978). The DABTH–amino acids were identified by thin-layer chromatography on 2.5 × 2.5 cm polyamide sheets (Schleicher & Schuell) (Chang, 1978) or by isocratic HPLC (Lehmann & Wittmann-Liebold, 1984). The standards for the identification of methylated alanine were prepared according to Chang (1978).

Results

Protein Components of Spinach Chloroplast Ribosomes. Two-dimensional polyacrylamide gel electrophoresis of spinach chloroplast r-proteins gives a distinctive separation pattern with about 50 spots (Figure 1). Previous immunoblotting experiments using antisera to purified, individual *E. coli* r-proteins (Bartsch, 1985) have shown that one of these spots contains a protein that is immunologically homologous to *E. coli* r-protein L2. Figure 1 shows the positions of this and 11 other chloroplast r-proteins which cross-react with specific *E. coli* antisera. They are designated after their *E. coli* counterparts on the basis of the immunological data. In the case of protein L12, the designation has been confirmed by primary structure data (Bartsch et al., 1982).

Purification of r-Protein L2. Purification of chloroplast r-proteins was made in a systematic manner as indicated under Materials and Methods. Figure 2A shows the elution and pooling profiles of Sephadex G-100 gel filtration chromatography. Pool A contained mainly nonribosomal proteins (e.g., ribulosebisphosphate carboxylase, which is a persistent contaminant in ribosome preparations) while pools B–E contained

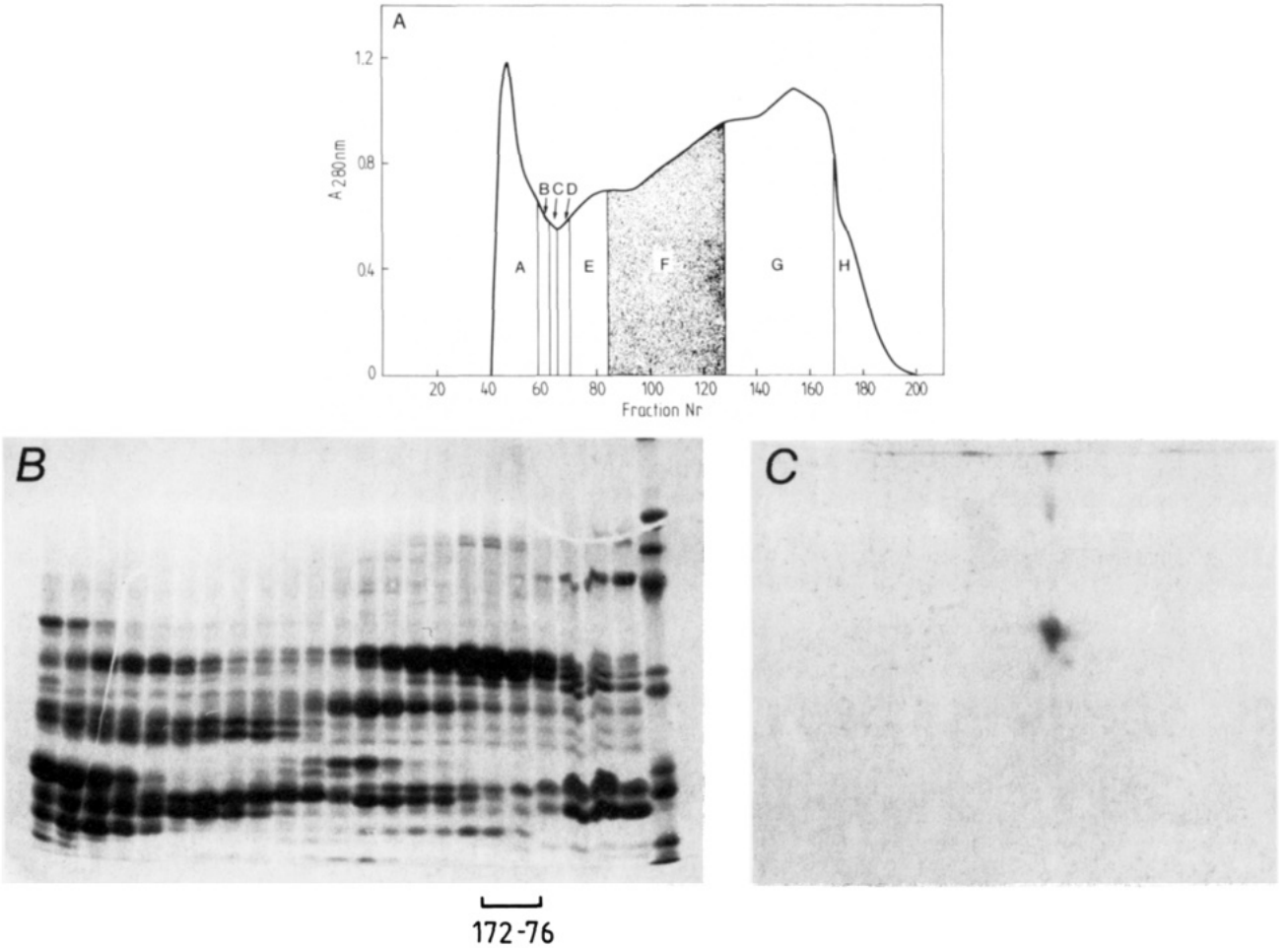

FIGURE 2: Partial purification of L2 by gel filtration and ion-exchange chromatography. Approximately 1 g of spinach chloroplast r-proteins was fractionated on a column of Sephadex G-100 (A). Pool F derived from it was chromatographed on a column of carboxymethyl-Sephadex, and fractions were analyzed by sodium dodecyl sulfate gel electrophoresis. Figure 2B shows the result for fractions 140–184 (every second fraction); those pooled for further purification of L2 are indicated. Figure 2C shows the two-dimensional gel electrophoresis of a sample of this pooled material.

relatively simple mixtures of the high molecular weight r-proteins. Pools F and G contained very complex and overlapping mixtures of the remaining r-protein components (pool H contained only traces of protein). Pool F was subjected to gradient chromatography on CM-Sephadex for further resolution of their components. Figure 2B shows SDS gel electrophoresis of the fractions (140–184) derived from the chromatography of pool F. The gel pattern of fractions 172–176 showed a major high molecular weight component, which was identified as r-protein L2 by two-dimensional gel electrophoresis (Figure 2C).

Further purification of L2 (as well as other chloroplast r-proteins) was made by reverse-phase HPLC (Materials and Methods) using pooled fractions from the CM-Sephadex chromatography and pools B–E from the gel filtration chromatography. The HPLC elution profile (Figure 3) of CM-Sephadex pool 172–176 showed a double peak as the major output. Two-dimensional gel electrophoresis of the material from the two peaks showed the same protein profile (i.e., L2). Amino acid analysis of the two peaks gave the same amino acid composition. Moreover, microsequencing of the two peak materials gave the same identical NH_2-terminal sequence. It should be noted that both *E. coli* and *Bacillus stearothermophilus* L2 are also known to elute in reverse-phase HPLC as multiple peaks (Kamp et al., 1984; Brockmöller & Kamp, 1986).

N-Terminal Sequence of r-Protein L2. The first 13 amino acid residues of spinach chloroplast L2, as determined by the

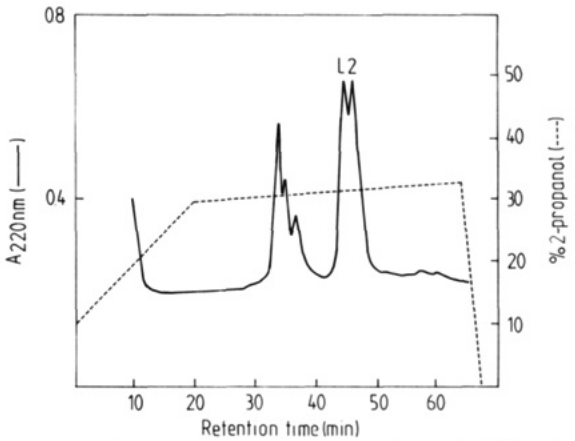

FIGURE 3: Purification of L2 by HPLC on a column of Vydac TP-RP. See text for data and discussion relating to the double peak of L2.

double coupling method, are NMAla[1]-Ile-His-Leu-Tyr[5]-Lys-Thr-Ser-Thr-Ser[10]-Ser-Thr-Arg[13]-. The N-terminal amino acid residue was *N*-methylalanine. It was identified according to two different methods.

In the first method, the DABTH–amino acid derivative obtained after the first degradation step was chromatographed two dimensionally on thin-layer sheets with two standards, namely, DABTH–alanine and DABTH–*N*-methylalanine, prepared from alanine and *N*-methylalanine, respectively. Figure 4A shows these chromatograms. It is evident that the

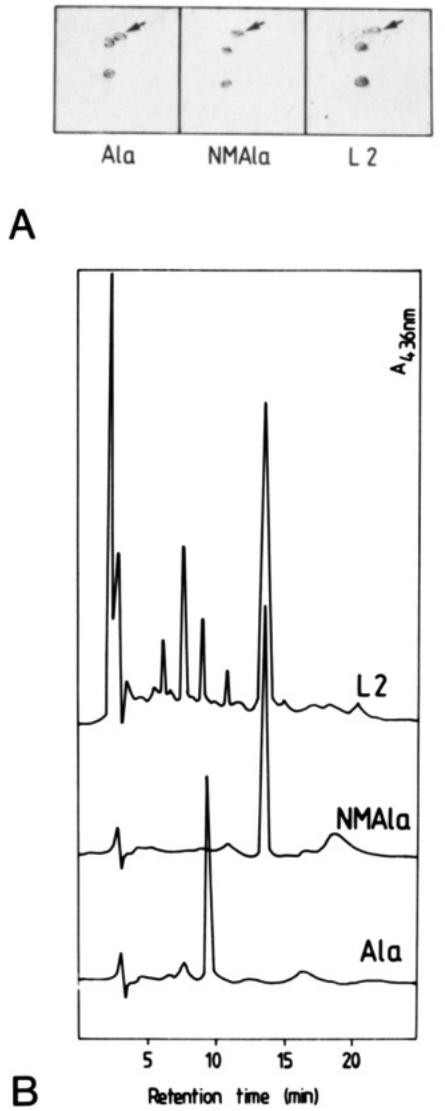

FIGURE 4: Separation of the DABTH derivatives of alanine, *N*-methylalanine, and that obtained from the N-terminal residue of spinach chloroplast L2 (A) by a two-dimensional thin-layer chromatographic procedure and (B) by HPLC separation.

amino acid derivative from protein L2 has the same migration characteristics as the derivative of *N*-methylalanine and not that of alanine.

In the second method, the two standards and the derivative obtained from protein L2 were separated on HPLC. The results show (Figure 4B) that the retention time for the DABTH derivative of the first amino acid residue of L2 is exactly the same as the retention time of DABTH–*N*-methylalanine.

From these experimental results and from those obtained with the CNBr fragments of L2 (see below), we conclude that the N-terminal amino acid of spinach chloroplast ribosomal protein L2 is *N*-methylalanine.

N-Terminal Sequences of the CNBr Fragments of r-Protein L2. The published putative amino acid sequence of spinach chloroplast r-protein L2, derived from nucleotide sequencing of a cloned chloroplast DNA fragment (Zurawski et al., 1984), has four methionine residues at positions 1, 122, 131, and 221. The molecule is 286 amino acid residues long. Cyanogen bromide cleavage of such a molecule should yield (excluding the first Met residue) four fragments of polypeptide chain length 121, 9, 90, and 65 residues. Figure 5 shows the HPLC separation of the polypeptide mixture obtained by CNBr

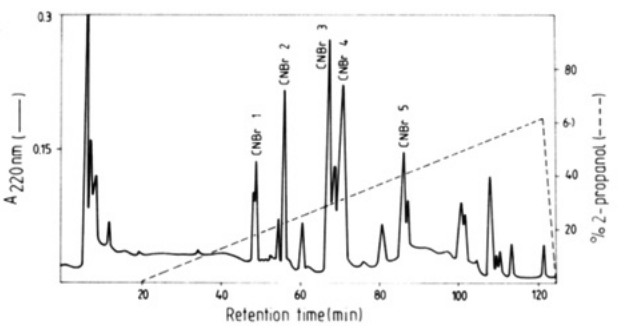

FIGURE 5: HPLC separation of the peptides obtained by CNBr cleavage of spinach chloroplast L2. N-Terminal amino acid sequences of the peptides in the peaks designated CNBr 1 to CNBr 5 were determined.

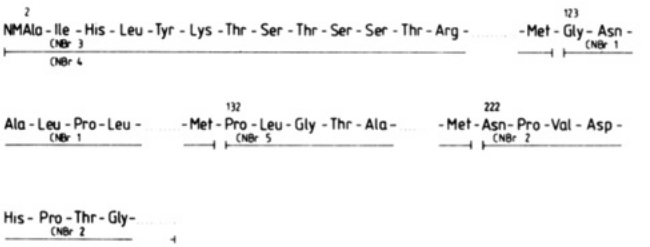

FIGURE 6: N-Terminal amino acid sequences of the five CNBr-derived peptides (Figure 5) of spinach chloroplast L2. The numbers shown refer to the residue numbers in the published amino acid sequence of spinach chloroplast L2 derived from nucleic acid sequencing (Zurawski et al., 1984).

cleavage of purified spinach chloroplast r-protein L2. Five of the peaks indicated in Figure 5 were collected, and the N-terminal sequences of the polypeptides contained in them were determined (Figure 6).

Two of the CNBr fragments (CNBr 3 and CNBr 4) yielded the N-terminal sequence identical with the N-terminal sequence of the intact protein. Both these fragments showed *N*-methylalanine as their N-terminal residue. Therefore, both are derived from the N-terminal part of the molecule and must have arisen from partial CNBr cleavage at the first methionine residue in the protein molecule. The remaining amino acid sequences of all four distinct CNBr peptides we obtained (including the expected positions of the methionine residues) showed perfect agreement with the amino acid sequence of spinach chloroplast L2 that has been derived from nucleotide sequencing (Zurawski et al., 1984).

Discussion

Protein Methylation in Chloroplast Ribosomes. The results presented in this paper throw light on three aspects of chloroplast ribosomes. First, it is evident from our results that methylation as a means of protein modification is maintained in chloroplast ribosomes. Since chloroplast evolution is believed to have taken place about 500×10^6 years ago or earlier (see the introduction), this result can be interpreted to mean that methylation of some ribosomal proteins serves an important function, although (as noted in the introduction) that particular function need not be essential for the basic steps in mRNA translation.

Ribosomal protein L2 of *E. coli* plays a role in the 50S ribosomal subunit assembly and in the peptidyltransferase reaction (Nierhaus, 1982). Yeast and archaebacterial ribosomes have been shown to contain a protein immunologically homologous to *E. coli* L2 (Schmid et al., 1984). *E. coli* L2 and *B. stearothermophilus* L2 are, however, nonmethylated proteins (Kimura et al., 1982, 1983). It thus appears likely that the pattern of individual r-protein methylation in chlo-

roplast ribosomes may be quite distinct from that in *E. coli*. In our previous structural work on chloroplast r-protein L12 (Bartsch et al., 1982), we did not find any evidence for methylation (or other modifications) of this chloroplast protein although protein L12 of *E. coli* is partially methylated (Terhorst et al., 1973). Thus, the feature conserved by evolution could be the methylation of certain r-proteins and not necessarily the methylation of homologous r-proteins.

Organelle Location of the L2 Gene. The assignment of identity to the chloroplast L2 gene sequence by Zurawski et al. (1984) was based entirely on the homology between its deduced amino acid sequence and the amino acid sequence of *E. coli* r-protein L2. Such a procedure has previously been used to identify maize chloroplast r-protein S4 (Subramanian et al., 1983) and tobacco chloroplast r-protein S19 (Sugita & Sugiura, 1983). The perfect agreement that exists between the 32 experimentally determined amino acid residue sequences (Figure 6) and the deduced sequence data of Zurawski et al. (1984) would argue that the latter is indeed the functional gene for spinach chloroplast r-protein L2. A similar conclusion regarding L2 has been drawn earlier from an in vitro transcription–translation analysis (Dorne et al., 1984).

Initiating Met Residue in Chloroplast-Synthesized Proteins. The total nucleotide sequences of tobacco and *Marchantia* chloroplast DNA (Shinozaki et al., 1986; Ohyama et al., 1986) have shown that they encode a complete set of tRNA genes including that for the intitiating $tRNA^{Met}$. However, the gene for a formylmethionine hydrolase has not therein been detected. The results reported in this paper indicate that such an enzyme activity is present in the chloroplast and it removes the formyl-Met residue from the N-terminus of newly synthesized chloroplast proteins such as L2. The gene for such an enzyme would probably be nucleus located.

Finally, it should be noted that *E. coli* L2, which is immunologically (Bartsch, 1985) and structurally (Zurawski et al., 1984; this paper) homologous to spinach chloroplast L2, has an unmodified Ala residue at the N-terminus. It may therefore serve as a substrate to detect and study the chloroplast L2 methylase enzyme. Since genes for any methylase enzymes have not been detected in the sequence of the total chloroplast DNA (Shinozaki et al., 1986; Ohyama et al., 1986), L2 methylase will also most probably be nuclear encoded.

Registry No. NM-Ala, 3913-67-5.

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