

Chloroplastic prenylated proteins

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Abstract By *in vivo* [³H]mevalonate labelling of spinach combined with biochemical analysis, evidence is provided for the existence of protein prenylation in chloroplasts. Approximately 20 prenylated polypeptides were resolved by SDS-PAGE followed by autoradiography. Thermolysin treatment of intact chloroplasts revealed that about 40% of the prenylated polypeptides were associated with the cytoplasmic surface of the outer envelope membrane. The remaining portion was present in thylakoids and/or the inner envelope membrane. The majority of the prenylated polypeptides were associated with larger membrane protein complexes. A farnesyl protein transferase activity was found to be associated with the thylakoid membrane.

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1. Introduction

Over the last decade, prenylated proteins have been studied extensively in mammalian tissue cultures and yeast, resulting in the identification of numerous proteins carrying an isoprenoid modification. These include rhodopsin kinase [1], small GTP-binding proteins [2,3], nuclear prelamin A and lamin B [4,5] and γ -subunits of heterotrimeric GTP-binding proteins [6]. Recently, evidence has been provided for the existence of prenylated proteins in plants [7], including small GTP-binding proteins [8,9] and DnaJ homologues [10,11].

Prenylated proteins identified to date were found to be modified by either farnesyl (C₁₅) or geranylgeranyl (C₂₀) moieties. In animals and yeast, four different enzymes catalysing protein prenylation have been identified: two farnesyl protein transferases [12,13] and two geranylgeranyl protein transferases [14,15]. These enzymes catalyse the formation of a thioether linkage between a cysteine residue, at or near the polypeptide carboxy terminus, and the isoprenoid molecule. In certain cases, prenylation occurs at two cysteine residues. The modifiable cysteine residues are found in specific carboxy terminal sequences: -CXC, -CC, -CCXX and -CXXX. Prenylation is often followed by additional protein modifications including proteolytic processing and carboxy methylation [16]. Recently, prenyl protein transferase activities have also been measured in several plant species [11,17,18]. Using molecular

biological techniques, it has been possible to identify several novel protein substrates for farnesylation in higher plants [19].

The physiological function of protein prenylation has not yet been fully established. However, it appears that prenylation is required for a particular set of proteins to exert their biological functions [20,21]. The current opinion is that prenylation facilitates membrane association of the modified protein due to the resultant increase in hydrophobicity. Protein prenylation also mediates protein-protein interactions [22].

In studies with tobacco cells in culture, the prenylation pattern was very similar to that of yeast and animal cells [17]. However, upon *in vivo* labelling of spinach with [³H]mevalonate, approximately 30 prenylated protein bands could be detected [7], far exceeding the number observed in non-photosynthetic eukaryotic cell cultures. It is possible that this difference is a consequence of *in vivo* labelling of a differentiated organism as compared to work performed in homogenous systems. Furthermore, the nature of prenylation in spinach was also found to differ considerably from that of yeast and animal cells [7,23]. These *in vivo* results imply the existence of unidentified prenylating enzymes, alternative target sequences and isoprenoid modifications other than farnesyl and geranylgeranyl groups.

In the present investigation, a combination of subcellular fractionation techniques and *in vivo* [³H]mevalonate labelling was used to study protein prenylation in spinach chloroplasts. Thus, for the first time both the localisation of prenylated proteins and farnesyl protein transferase activity within the chloroplast have been demonstrated.

2. Materials and methods

(*R,S*)-[5-³H]Mevalonolactone (specific activity 2.8–12.5 Ci/mmol) was prepared as described by Keller [24]. All-*trans*-farnesyl (specific activity 0.36 Ci/mmol) pyrophosphate was prepared as described earlier [25]. DL-Dithiothreitol (DTT) was ultrapure grade. Mevinolin was the generous gift of Dr. A.W. Alberts (Merck). Before use, the mevalonolactone and mevinolin were converted to their metabolically active forms as described previously [7]. All chemicals were obtained from Sigma and all solvents were of reagent grade.

Spinach seedlings (*Spinacea oleracea* L.) were grown on vermiculite in a growth chamber at 22–25°C, with a 10 h photoperiod for 2–3 weeks. Leaf pieces (approx. 5×5 mm) were cut and floated in small Petri dishes (0.5 g/dish) on labelling medium [7] containing 30 μ M mevinolin and 0.05% Tween-20. A preincubation, under laboratory illumination, was performed for 20 h prior to the initiation of mevalonate labelling, to allow the inhibition of hydroxymethylglutaryl-CoA reductase activity [26]. Labelling was then performed for 24 h with 0.5 mCi [³H]mevalonate in 0.5 ml medium.

Intact chloroplasts were isolated from 3 g of mevalonate-labelled leaf pieces on a discontinuous Percoll gradient according to standard procedures [27]. Intact chloroplasts were, when indicated, treated with thermolysin (750 μ g/mg chlorophyll) [28] in the presence of 0.5 mM CaCl₂ for 30 min in the dark on ice. The reaction was stopped with

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10 mM EDTA and intact chloroplasts were then re-isolated on a discontinuous Percoll gradient.

From the [^3H]mevalonate labelled samples, chloroplasts were lysed in 5 mM MgCl_2 , 15 mM NaCl, 20 mM MES (pH 6.3) and the stromal contents separated from the membrane fraction by centrifugation at $5000\times g$ for 10 min. The proteins in each fraction were then precipitated with 10% trichloroacetic acid (TCA) (>30 min on ice) after which they were extracted twice with a series of organic solvents to remove non-covalently bound mevalonate metabolites and solubilised as described previously [18]. Protein concentrations were determined according to Marder et al. [30], using bovine serum albumin as the standard. An aliquot of each radiolabelled sample was removed for scintillation counting. Crude fractionation of intact chloroplasts isolated from unlabelled leaves was basically performed according to Hirsch and Soll [29]. Chloroplasts were lysed as above and the thylakoid fraction was isolated by centrifugation at $1500\times g$ for 10 min. The supernatant was subjected to ultracentrifugation at $150\,000\times g$ for 90 min to pellet envelopes. The remaining supernatant was collected and used as the stromal fraction.

Chloroplast membrane protein complexes were separated by sucrose gradient centrifugation according to the method of van Wijk et al. [31,32]. After centrifugation, the gradients were divided into 600 μl fractions and the proteins immediately precipitated by the addition of TCA to a final concentration of 10%. The precipitated proteins were extracted once with 80% acetone and solubilised. The protein profiles of the samples were resolved on 13% acrylamide gels containing 6 M urea which were run at 12°C [33]. Gels were stained with Coomassie blue R250, then incubated in Amplify (Amersham, UK) for 20 min prior to drying. Autoradiography was performed for as long as 4–10 weeks at -80°C with Hyperfilm-MP (Amersham). Radiolabelled molecular mass (Rainbow) markers were obtained from Amersham.

Farnesyl protein transferase activity was assayed in chloroplast fractions by measuring the amount of [^3H]farnesyl transferred from [^3H]farnesyl pyrophosphate to dithiothreitol in polypropylene tubes [18], with the addition of 0.05% *n*-octyl β -D-glucopyranoside in each reaction mixture. Protein content was estimated according to Lowry et al. [34]. When preparing fractions for farnesyl protein transferase activity measurements, MgCl_2 and EDTA were omitted from all the buffers.

Coding sequences for chloroplast sequences in the Genpept database were searched for the C-terminal motifs -CXXX, -CC and -CXC.

3. Results and discussion

In a previous study it was established that plant organelles contain a number of prenylated proteins [7]. This finding raised several questions and in the present study we have specifically addressed the issue of protein prenylation in the chloroplast. To this end, spinach leaves were labelled with [^3H]mevalonate for 24 h and intact chloroplast were isolated. The purity of the chloroplast preparation was assessed by transmission electron microscopy, which revealed that the preparation was essentially free of contamination from other organelles such as mitochondria, nuclei, microsomes or other membrane fragments, and that the chloroplasts were more

Table 1
Distribution of prenylated proteins within the chloroplast

Chloroplast fraction	Incorporation of radiolabel (dpm/ μg protein)
Membranes	850 ± 260
Stroma	35 ± 15

Intact chloroplasts were isolated from leaves labelled with [^3H]mevalonate and subfractionated. Proteins in each fraction were precipitated with TCA and extracted twice with each of the following solvents: acetone, chloroform:methanol (2:1), 95% ethanol, and chloroform:methanol:water (1:1:0.3). The proteins were then solubilised for SDS-PAGE. An aliquot of each fraction was removed for scintillation counting and protein determination. Numbers shown are means of four experiments \pm S.D.

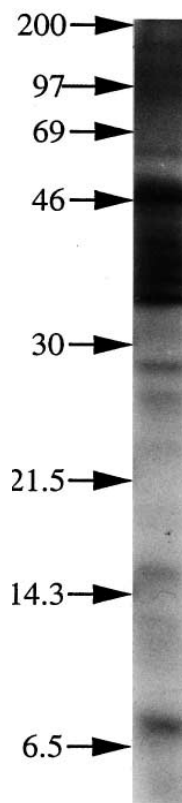


Fig. 1. Autoradiograph of prenylated proteins in intact chloroplasts isolated from spinach leaves *in vivo* labelled with [^3H]mevalonate. The chloroplasts were prepared on a Percoll gradient. The proteins were precipitated, lipid extracted and subjected to SDS-PAGE.

than 85% intact. To minimise unspecific adsorption of cytoplasmic proteins to the chloroplasts, they were washed repetitively with alkaline medium. Furthermore, it has been shown previously [7] that the cytosol of spinach seedlings contains very few, if any, proteins which can be labelled with [^3H]mevalonate.

Total chloroplast protein was precipitated, subjected to extensive lipid-extraction and subsequently analysed by SDS-PAGE combined with autoradiography (Fig. 1). The results revealed that a number of chloroplastic polypeptides contain covalently bound mevalonate metabolites, consistent with the presence of prenylated proteins in this organelle. There are at least 10 major radioactive bands but, in total, approximately 20 labelled polypeptides could be detected. The majority of the radioactivity was associated with polypeptides in the 35–46 kDa molecular mass range but proteins of low molecular mass were also found to be labelled, such as a dominant 8 kDa polypeptide (Fig. 1).

In the next set of experiments, we analysed if the chloroplastic prenylated proteins are soluble stromal proteins or are membrane bound. The intact chloroplasts were lysed by osmotic shock and the membranes were separated from the stroma by centrifugation. As can be seen in Table 1, the vast majority of the covalently bound radiolabel was found to be membrane-associated. On a protein basis, labelling of the membrane fraction was at least 25-fold greater than of the stroma fraction.

The membrane fraction is dominated by the abundant thylakoids but its content of chloroplast envelopes should not be

neglected. A straightforward subfractionation experiment involving analyses of isolated envelope membranes and thylakoids was not realistic from an experimental point of view. The *in vivo* labelling experiment maximally allows handling of 3 g of spinach leaves, excluding the possibility of isolating pure envelope membranes in amounts required for accurate analysis. Therefore, in order to analyse the occurrence of prenylated proteins in the chloroplast envelope, a more indirect approach was applied. The intact chloroplasts were subjected to proteolytic thermolysin treatment and then re-isolated by centrifugation. Following such a treatment the relative amount of covalently bound radiolabel could be reduced by as much as 42% compared to the untreated control chloroplasts (Table 2). This observation gives strong support to the contention that a substantial proportion of chloroplast prenylation is found at the cytoplasmic side of the outer envelope membrane. These prenylation reactions are likely to be catalysed by prenyltransferases located in the cytosol of the plant cell. The remaining 58% of radioactive labelling, resistant to the thermolysin treatment, should represent prenylated proteins mainly associated with the inner chloroplast envelope membrane or the thylakoid membrane. A direct experimental discrimination between these two possibilities is hampered by the limitations for subfractionation of *in vivo* labelled chloroplasts (but see experiment described in association with Table 3 below).

The prenylated membrane-polypeptides were studied by analytical sucrose gradient centrifugation following solubilisation by *n*-dodecyl β -D-maltoside. This procedure enables fractionation of membrane proteins or membrane protein complexes according to molecular mass in a calibrated manner [31,32]. Fig. 2A illustrates that prenylated polypeptides were recovered throughout the sucrose density gradient. Thus, prenylated polypeptides are found in entities of molecular masses ranging from 30 kDa to 500 kDa. The autoradiogram of Fig. 2B shows that fraction 4–5 contained both low molecular mass (8 kDa) and high molecular mass (88 kDa) radiolabelled polypeptides. In addition these fractions, which represent very large protein complexes, contained prominent radiolabelled bands at 22 and 24 kDa. Fractions 8–11, which contain complexes of 80–250 kDa, were dominated by a polypeptide at 33 kDa plus some diffuse bands of relatively high molecular masses. The smaller membrane complexes of fractions 12–14 contained a distinct low molecular mass radiolabelled polypeptide in addition to polypeptides of 22, 24 and 56 kDa. The top fractions, where extrinsic membrane proteins are recovered, did not show any prominent radiolabelled bands. The centrifugal analysis revealed that the majority of the prenylated proteins are part of larger membrane protein complexes or present as homo-multimers. The co-sed-

Table 2
Thermolysin digestion of chloroplastic prenylated proteins

Thermolysin digestion	Labelling (% of non-digested chloroplasts)
–	100
+	58 \pm 5

Chloroplast were isolated from spinach seedlings. An aliquot was incubated with thermolysin (750 μ g/mg chlorophyll) for 30 min on ice before being re-isolated in the presence of 10 mM EDTA. The chloroplast proteins were then treated as described in Table 1. Numbers shown are means of three experiments \pm S.D.

Table 3
Farnesyl protein transferase activity within the chloroplast

Chloroplast fraction	Activity (pmol/mg protein/min)
Envelopes	0.423 \pm 0.22
Thylakoids	16.7 \pm 3.7
Stroma	–

Chloroplast subfractions were isolated from spinach seedlings and incubated with DTT and [3 H]FPP in polypropylene tubes. The amount of product formed was determined by scintillation counting. Controls, omitting DTT, were performed for each measurement and subtracted as background. Numbers shown are means of four experiments \pm S.D.

imentation of radiolabelled proteins with the pigmented bands I–III (Fig. 2A), which represent photosystem I and photosystem II complexes, could indicate an association of prenylated proteins with the photosynthetic apparatus although such an interpretation would require further experimentation. However, from silver stain analysis of SDS-PAGE it could be concluded that none of the prenylated proteins are abundant (data not shown). One should also bear in mind that a substantial fraction of the radiolabelled bands are not of thylakoid, but of envelope membrane origin.

Protein prenylation in chloroplasts was not only analysed at the substrate, but also at the enzyme level. Following subfractionation of chloroplast from unlabelled spinach leaves, allowing isolation of envelopes, thylakoid membranes and stromal fractions, each of the three fractions was analysed with respect to farnesyl protein transferase activity. As shown in Table 3, only the thylakoid fraction possessed any significant catalytic ability when DTT was used as a farnesylation substrate. Only a very low activity was found in the envelope fraction while the stroma did not show any farnesylation activity. Thus, the major portion of chloroplastic farnesyl protein transferase activity is associated with the thylakoid membrane. Localisation of the enzymatic activity to the soluble thylakoid lumen was excluded by prenylation studies using a lumen fraction purified from spinach thylakoids (T. Kieselbach and I. Parmryd, unpublished observation).

In conclusion, the results obtained give strong evidence that post-translational prenylation of chloroplast proteins does take place. A portion of these prenylation reactions appears to take place outside the actual organelle and involves proteins at the cytoplasmic surface of the outer envelope membrane. However, prenylation must also occur inside the chloroplast as judged by the existence of protein labelling inaccessible to thermolysin proteolysis of intact organelles and to the presence of farnesyl protein transferase in the thylakoid fraction. The latter finding is somewhat surprising since prenyl protein transferases normally are soluble and not membrane-bound [16]. At present, we cannot discriminate between the possibilities of whether the farnesyl protein transferase is attached to the thylakoid surface or an integral part of the membrane. The association of the enzymatic activity with the thylakoids suggests the presence of prenylated proteins in the photosynthetic membrane. We do not have direct evidence for prenylation of proteins associated with the inner envelope membrane, so this possibility can not be excluded. So far, no information is available with respect to geranylgeranyl protein transferase activity in chloroplasts.

The present work raises several questions related to the physiological function of chloroplast protein prenylation as

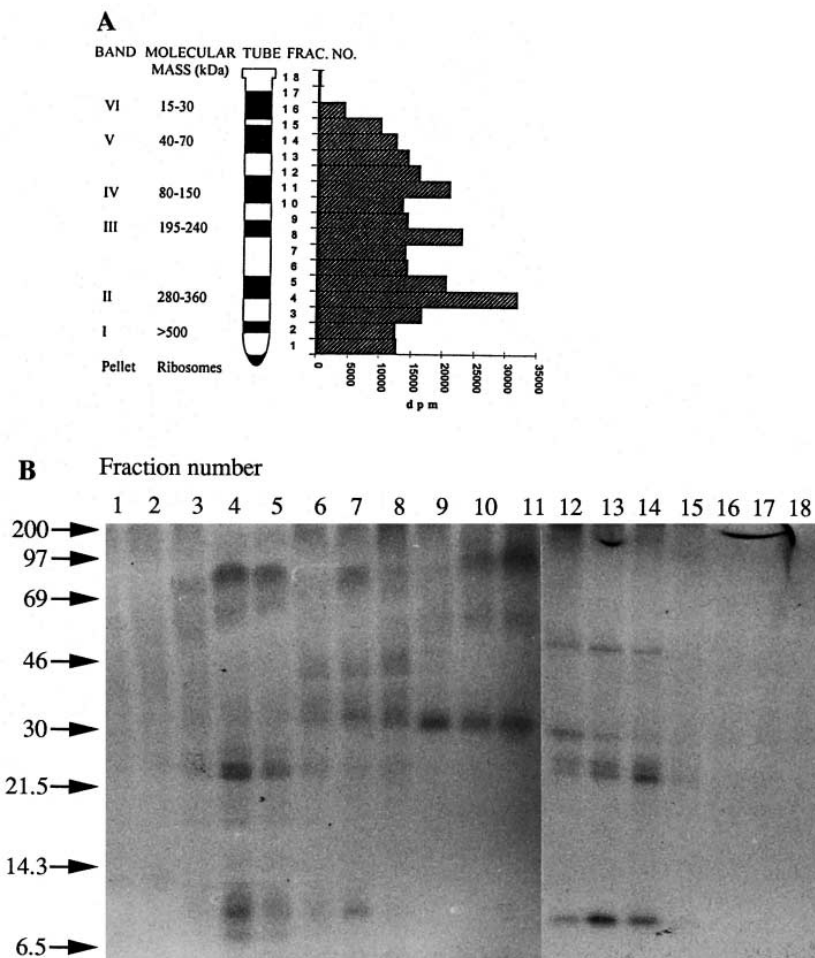


Fig. 2. Intact chloroplasts from leaves labelled with [3 H]mevalonate were isolated, lysed and fractionated by sucrose density centrifugation. Proteins in each gradient fraction were precipitated with TCA, washed with 80% acetone and solubilised for SDS-PAGE. An aliquot of each fraction was removed for scintillation counting and protein determination. A: Diagrammatic representation of a sucrose density centrifugation tube as observed after centrifugation of the detergent-solubilised chloroplast membrane fraction showing the pigmented bands obtained, their approximate molecular masses, and the protein-bound radioactivity associated with each gradient fraction. B: Autoradiograph of the labelled polypeptides present in the gradient fractions. The position of molecular mass markers (kDa) is shown to the left.

well as to the identity of the modified proteins. It is possible that the 8 kDa polypeptides represent γ -subunits of heterotrimeric GTP-binding proteins which characteristically have molecular masses of 5–8 kDa in other organisms [35–37]. Moreover, the 22–26 kDa prenylated polypeptides have molecular masses representative of small GTP-binding proteins [38]. UV cross-linking experiments have indicated that several chloroplast polypeptides are capable of binding GTP (C.A. Shipton, unpublished observation), and chloroplast GTP-binding proteins have previously been identified in both the thylakoid and envelope membranes [39–42].

A search of the translated DNA coding regions for chloroplast-encoded or nuclear-encoded chloroplast proteins revealed that several identified proteins and unknown coding regions contain currently recognised prenylation motifs. Among the known proteins were several *ndhF*, *rps8* and *rbcL* gene products and *sce70* (a heat-shock protein homologue). It is distinctly possible that the chloroplast prenylated proteins observed in the present study possess prenylation sequences which are not yet recognised since it has been reported that plant prenyl transferases have different sequence specificities than their yeast and mammalian counterparts [43].

Furthermore, considering the versatility of the mevalonate pathway in plants compared to other organisms one should consider that plants may well possess isoprenoid modifying groups other than farnesyl and geranylgeranyl. In support of this possibility is the release of unidentified isoprenoids upon hydrolysis of labelled plant proteins [7,23].

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