Short Communication

(-) S-adenosyl-L-methionine-magnesium Protoporphyrin Methyltransferase, an Enzyme in the Biosynthetic Pathway of Chlorophyll in Zea mays

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Summary. The enzyme (-) S-adenosyl-L-methionine-magnesium protoporphyrin methyltransferase, which catalyzes the transfer of the methyl group from (-) S-adenosyl-L-methionine to magnesium protoporphyrin to form magnesium protoporphyrin monomethyl ester, has been detected in chloroplasts isolated from Zea mays. Protoporphyrin and free protoporphyrin also act as substrates in the system, although neither one is as active as magnesium protoporphyrin.

The following scheme of chlorophyll synthesis in higher plants is proposed: 
δ-aminolevulinic acid → → protoporphyrin → magnesium protoporphyrin → magnesium protoporphyrin monomethyl ester → → chlorophyll a.

though the formation of magnesium protoporphyrin methyl ester from magnesium protoporphyrin seems the more likely course the slight possibility exists that protoporphyrin may be the substrate in vivo and that in R. spheroides magnesium may be introduced into the porphyrin after methylation.

The present study was undertaken to study porphyrin methylation in plastids from leaves of green plants.

Material and Methods

Magnetism protoporphyrin and zinc protoporphyrin were made according to the method of Baum, Burnham, and Plane (1). The protoporphyrin was the commercially available grade which was further purified by counter-current distribution. (-) S-adenosyl-L-methionine-Me¹⁴C and (-) S-adenosyl-L-methionine were obtained from Cal Biochem; the labeled (-) S-adenosyl-L-methionine was diluted to a specific activity of 1 μc/μmole before use.

Zea mays was grown on vermiculite in the dark for 10 to 12 days at a temperature of 20 to 25°. Before being used the etiolated plants were illuminated with about 150 ft-c of fluorescent light for 4 hours at 25 to 30°. The plants were then harvested and 100 grams of leaves were ground with sand in a mortar with 200 ml of a solution consisting of 0.5 M sucrose, 0.5 M tris-HCl pH 8, and 0.001 M MgCl₂. This suspension was filtered through 3 layers of muslin and centrifuged for 5 minutes at 120 × g to remove cell debris. The supernatant was then centrifuged for 8 minutes at 1000 × g to spin down the chloroplasts. The chloroplast pellet was suspended in 5 ml of 0.05 M

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tris buffer pH 8. This suspension was used as a crude enzyme preparation.

The assay for (−) S-adenosyl-L-methionine-magnesium protoporphyrin methyltransferase activity was a modification of the procedure used by Gibson, Neuberger, and Tait (2). The reaction mixture contained 0.5 ml of crude extracts (0.96 to 1.11 mg soluble protein per ml), 0.1 μmole porphyrin or metalloporphyrin, and 0.5 μc of (−) S-adenosyl-L-methionine-Mel 14C in a total volume of 1 ml. The mixture was incubated for 2 hours at 37°.

Enzymatic activity was assayed by following the incorporation of radioactivity from (−) S-adenosyl-L-methionine-Mel 14C into the total porphyrin fraction. After incubation, the porphyrins were extracted with 5 ml of ethyl acetate-acetic acid (3:1, v/v). This mixture was centrifuged and the precipitate washed again with 5 ml of ethyl acetate-acetic acid. After centrifugation the combined supernatants were neutralized with 6 ml of saturated sodium acetate. After discarding the aqueous phase the ethyl acetate solution was washed twice with 10 ml of water. Porphyrins were extracted from this solution with 5 ml of 3 N HCl. This HCl extract was then washed with 6 ml of ethyl acetate. Four ml of fresh ethyl acetate was then layered over the HCl and the porphyrins were driven into the ethyl acetate layer by neutralizing the HCl with solid NaHCO3. The aqueous layer was then discarded. Two ml of methanol were added and the solution then washed twice with 8 ml of water. This ethyl acetate solution of porphyrins was then used for analysis. No detectable radioactive substrate was carried through the purification (table I).

When the porphyrins were extracted with the ethyl acetate-acetic acid mixture the magnesium and zinc porphyrins were converted to the corresponding free porphyrins. All subsequent determinations were made on metal-free porphyrins. It was assumed that the porphyrins and their monoesters behave in identical fashion during the extraction, and that the relative amounts are the same in the final ethyl acetate solution as they are at the end of incubation.

The concentration of the porphyrins was estimated by extracting the porphyrins contained in 0.1 ml of the ethyl acetate into 2 ml of 3 N HCl and reading the optical density at 408 μ (ε4MN = 262 for protoporphyrin) using a Beckman DU Spectrophotometer.

The radioactivity of the sample was determined by transferring a measured amount of the ethyl acetate solution (100 μl) into a scintillation counting vial, adding 10 ml of scintillation fluid (7) to each vial, and counting in a Packard Tri-Carb Scintillation Spectrometer. A known amount of radioactivity i.e. 375 DPM of 14C-toluene, was then added, and the vials counted again to permit a correction for quenching to be made. A radioactive standard was also counted to determine the efficiency of the scintillation counter for 14C; this enabled the results to be calculated as disintegrations per minute.

The ethyl acetate solutions of the porphyrins were analyzed by descending paper chromatography. The chromatograms were developed for 24 hours in 2.6 lutidine, 1.08 x ammonia, water, and 1 M EDTA (25:10.5:7:0.05, v/v). The chromatogram was then cut into 2 cm squares, and the squares were counted in the scintillation counter using 10 ml of scintillation fluid per vial (7).

Hydrolysis of porphyrin methyl ester was accomplished by treating the pigment contained in 100 μl of the ethyl acetate solution with 1 ml of 8 N HCl at 37° for 4 hours. The HCl was then evaporated under reduced pressure. The residue was taken up in 0.5 ml of Hyamine (11), and 10 ml of scintillation fluid were added. The samples were then counted in the scintillation counter.

Results

The results of the methyltransferase experiments are summarized in table I. Magnesium protoporphyrin was the best of the 3 substrates provided although both protoporphyrin and zinc protoporphyrin were esterified to limited extents. In each experiment a control was run which contained no substrate. The radioactivity of the control was taken as a measure of the effectiveness of the extraction procedure for removing labeled substrate.

<p>| Table I. Enzymatic Incorporation of 14C from (−) S-adenosyl-L-methionine-methyl 14C into Porphyrins |
|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Porphyrin substrate</th>
<th>Specific activity of products DPM/μmole of porphyrins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin</td>
<td>990</td>
</tr>
<tr>
<td>Magnesium protoporphyrin</td>
<td>7320</td>
</tr>
<tr>
<td>Expt 1 Zinc protoporphyrin</td>
<td>3250</td>
</tr>
<tr>
<td>No substrate</td>
<td>nil</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td>740</td>
</tr>
<tr>
<td>Magnesium protoporphyrin</td>
<td>6520</td>
</tr>
<tr>
<td>Expt 2 Zinc protoporphyrin</td>
<td>1720</td>
</tr>
<tr>
<td>No substrate</td>
<td>nil</td>
</tr>
</tbody>
</table>

Paper chromatography of the porphyrins showed all of the radioactivity to be in the spot (Rf ca. 0.88) moving as a porphyrin with 1 free carboxyl group.

The radioactivity of the purified porphyrin fraction was completely eliminated by treatment with 8 N HCl. This behavior is consistent with the incorporation into a methyl ester group and
not into an N-methyl, C-methyl, or methoxyl group.

Discussion

This system did not produce enough magnesium protoporphyrin methyl ester to permit unequivocal identification of the product by determination of its HCl number etc., but similar work by Tait, Gibson, and Neuberger (2,12) and the large differences in reaction rates with the substrates tested seem to justify the assumption that the main reaction catalyzed by the corn chloroplast enzyme is indeed: magnesium protoporphyrin \( \rightarrow \) magnesium protoporphyrin monomethyl ester.

The zinc protoporphyrin seems to serve as substrate for esterification although it is less satisfactory than magnesium protoporphyrin; no zinc porphyrins are known to occur naturally. The esterification of the zinc porphyrin may also help to explain the incorporation of label when metal-free porphyrin is included in the incubation mixture. Zinc is known to be chelated non-enzymatically by protoporphyrin (9) and a zinc porphyrin chelatase has been shown to exist in higher plants (8). Therefore, it is possible that the porphyrin which was methylated was in fact zinc protoporphyrin. Another alternative is that this enzyme may be unspecific enough to catalyze the methylation of protoporphyrin directly. In any event, of the 3 porphyrins tested, magnesium protoporphyrin was clearly methylated most actively.

The differences in reaction rates between the 2 experiments can probably be attributed to the differences in the plastid preparation used. The isolation procedures are subject to large variations owing to differences in \( \text{H}_2\text{O} \) content of the leaves, loss during extraction, etc. Therefore, valid comparisons can only be made within each experiment. The differences in zinc protoporphyrin incorporation are not so easily explained, however.

These experiments indicate that this enzymatic system in Zea mays is similar to that in \( \text{R. spheroides} \). A methyltransferase system has also been detected in \( \text{Phaseolus vulgaris} \) which exhibits roughly the same properties (10). The results indicate that chlorophyll synthesis in this system proceeds according to the scheme: \( \delta \)-aminolevulinic acid \( \rightarrow \rightarrow \) protoporphyrin \( \rightarrow \) magnesium protoporphyrin \( \rightarrow \) magnesium protoporphyrin monomethyl ester \( \rightarrow \rightarrow \) chlorophyll a.

Literature Cited