ESTERIFICATION OF CHLOROPHYLLIDE BY GERANYLGERANYL PYROPHOSPHATE IN A CELL-FREE SYSTEM FROM MAIZE SHOOTS

W. Rüdiger, P. Hedden, H.-P. Köst and D. J. Chapman

Botanisches Institut, Universität München, West Germany,

and Department of Biology, University of California,

Los Angeles, Calif. 90024 USA

Received November 19,1976

SUMMARY: A cell-free system is described which incorporates [^{14}C]-geranylgeranyl pyrophosphate, but not free [^{14}C]-geranylgeraniol, into chlorophyll $a_{geranylgeraniol}$. The esterifying enzyme is found in the 75,000 g pellet of a homogenate from maize shoots whereas most of the phosphatase activity remains in the supernatant. The enzyme is different from chlorophyllase which has been discussed in the literature as the possible esterifying enzyme.

INTRODUCTION: The last part of chlorophyll biosynthesis in higher plants - after photoconversion of protochlorophyllide to chlorophyllide a - is the phytyl ester formation. Most authors consider this step to be a chlorophyllase catalyzed esterification of chlorophyllide (1,2,3) or methyl chlorophyllide (2-5) with free phytol. This reaction, which has been studied *in vitro*, requires high concentrations of an organic solvent (e.g. acetone) and a large excess of free phytol which is not found in greening leaves (6). The suggestion that phytyl pyrophosphate is the substrate for phytylation (7) has been rejected mainly because chlorophyllase will not utilize this compound (5). Geranylgeranyl pyrophosphate is also not a substrate for this enzyme (5). Results from recent kinetic (8,9) and inhibitor (10) experiments suggest that phytylation is a multi-step reaction: Chlorophyllide geranylgeranyl ester is formed first and is then hydrogenated stepwise to the phytyl ester. We describe here a cell-free system which catalyzes the formation of chlorophyllide geranylgeranyl ester.

MATERIALS AND METHODS: Maize seeds (*Zea mays* L., cv. Perdux, Bayer. Futtersaatbau-Vereinigung, München) were soaked for 24 h in water and then allowed to germinate for 4 days at 27° in the dark on moist Vermiculite. The etiolated shoots were cut 1-2 cm below the apex under green safelight and the shoot tips irradiated for 1 min with white incandescent light (2000 lux) followed immediately by freezing in liquid nitrogen. All subsequent procedures were performed under green safelight. The frozen shoots (7 g) were ground to a fine powder in a

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

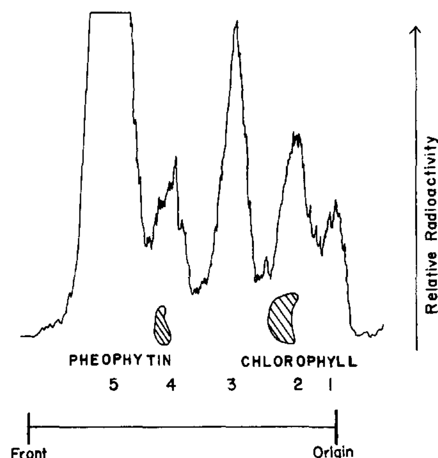

Figure 1. Radiochromatogram from thin-layer chromatography of the products after incubation of the 75,000 g pellet from homogenates of maize shoots with [^{14}C]-geranylgeranyl pyrophosphate. Silica gel G developed first with CCl_4/acetone (20:1) to 10 cm followed by petroleum ether/ethyl acetate/pyridine (58:30:12) to 6 cm. Zone 1- origin; 2- chlorophyll a_{GG}; 3- geranylgeraniol; 4- pheophytin a_{GG}; 5- hydrocarbons.

---

pre-cooled mortar. Polyclar AT (1 g) was then added followed by 8 ml of 0.1 M phosphate buffer (pH 7.0) containing 5 mM mercaptoethanol, and the mixture ground further to a slurry. Large cell debris were removed by centrifugation at 2000 g for 10 min. A particulate fraction was obtained by centrifugation at 75,000 g for 1 h. The pellet was resuspended in the same buffer (3 ml) and centrifuged again at 75,000 g. The washed pellet was resuspended in the same buffer (3.5 ml).

[^{14}C]-Geranylgeranyl pyrophosphate (20 µCi/µmole) was prepared biochemically from [2-^{14}C]-mevalonic acid (5 µCi/µmole) using a cell-free system from endosperm of immature seeds of *Echinocystis macrocarpa* Greene, as described by Oster and West (11). The [^{14}C]-geranylgeranyl pyrophosphate (1.2×10^5 dpm, 2.8 nmoles) was incubated with 1 ml of the 75,000 g pellet suspension containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$ for 2 h at 30° . The incubation was stopped by the addition of acetone (1 ml) and the pigments were extracted with ethyl acetate (3×1 ml). After TLC of a fraction of the extract, the plate was scanned for radioactivity (figure 1). The remainder of the ethyl acetate extract was treated with 5% HCl (0.1 ml) and, after washing with water and drying over Na_2SO_4, subjected to TLC.

RESULTS: After a two hour incubation of the 75,000 g pellet of a homogenate from maize shoots with [^{14}C]-geranylgeranyl pyrophosphate, the ethyl acetate extract contains 20-25% of the total radioactivity. Typically the distribution of radioactivity after TLC of this extract is as shown in figure 1. We observe five radioactive zones: 1 - the radioactivity at the origin

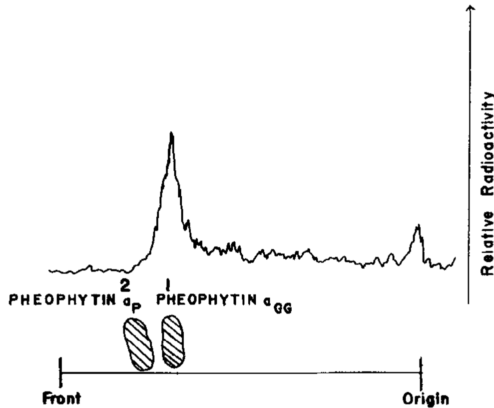

Figure 2. Rechromatography of zone 4 (see figure 1) on silver nitrate impregnated silica gel G developed with CCl_4/acetone (9:1). 1- pheophytin a_{GG} (pheophorbide geranylgeranyl ester) which migrates with the radioactivity. 2- pheophytin a_p (pheophorbide phytyl ester) which contains no radioactivity.

---

varies with the effectiveness of washing and drying the organic phase and is presumably unreacted substrate. 2 - this zone runs slightly behind chlorophyll a and is identified as chlorophyll a_{GG} (chlorophyllide geranylgeranyl ester) (see below). 3 - free geranylgeraniol formed by the action of phosphatases on the substrate. It was identified by co-chromatography with an authentic sample and with the product formed by treatment of [^{14}C]-geranylgeranyl pyrophosphate with alkaline phosphatase in benzene/ethyl acetate 9:1 (R_f 0.4) and in carbon tetrachloride/acetone 20:1 (R_f 0.25). 4 - this zone runs slightly behind pheophytin a and is identified as pheophytin a_{GG} (pheophorbide geranylgeranyl ester) (see below). 5 - hydrocarbon fraction (containing e.g. carotenes) which was not further identified.

After treatment of the organic phase with HCl both the chlorophyll and the radioactive zone 2 are lost. There is then an increase in pheophytin a and also in the radioactivity in zone 4. The transformation of chlorophyll into pheophytin was also carried out with the material eluted from zone 2 in figure 1. Upon rechromatography of this material in carbon tetrachloride/acetone 20:1 and in benzene/ethyl acetate 9:1 the radioactivity migrates as a single zone just behind the added carrier pheophytin. This product co-chromatographs with pheophytin a_{GG} (pheophorbide geranylgeranyl ester, prepared as previously described (10)) on a silver nitrate impregnated plate (figure 2), but not with pheophytin a_p (pheophorbide phytyl ester).

Incubation of the 75,000 g pellet with [^{14}C]-geranylgeraniol, rather than its pyrophosphate, results in no incorporation of radioactivity into the chlorophyll or pheophytin fraction. Incubation of the 75,000 g supernatant with [^{14}C]-geranylgeranyl pyrophosphate yields free geranylgeraniol as the main product (due to phosphatase activity) but no labeled chlorophyll or pheophytin.

DISCUSSION: The cell-free system described here contains the enzyme responsible for the esterification of endogenous chlorophyllide. Although it has not been fully characterized, the known properties can clarify some of the controversies found in the literature. The enzyme utilizes geranylgeranyl pyrophosphate as substrate but not the free alcohol. Therefore (and because it does not need acetone in the medium) it is different from chlorophyllase. The enzyme has been separated here from most of the phosphatase activity which is found in the 75,000 g supernatant. The incorporation is much lower in incubations with whole homogenates because of the rapid formation of free geranylgeraniol which is not incorporated. The participation of phosphatases may explain why the incorporation of isopentenyl pyrophosphate (12) and phytyl pyrophosphate (13) into the phytyl residue of chlorophyll in bean homogenates was 1-2 orders of magnitude lower than that of geranylgeranyl pyrophosphate in our experiments. We obtained routinely an incorporation of approximately 1% of the radioactivity from geranylgeranyl pyrophosphate into chlorophyll and pheophytin (5% of the ethyl acetate extractable radioactivity). It may also be possible that the enzyme is specific for geranylgeranyl pyrophosphate so that geranylgeraniol (8,9,10) and not phytol is the natural esterifying moiety. Investigation of the substrate specificity and the further purification of the enzyme are under way.

ACKNOWLEDGEMENTS

We thank Professor West for a gift of geranylgeraniol, Mr. J. Benz for pheophytin a_{GG} and silver nitrate plates. W. Rüdiger and H.-P. Köst thank the Deutsche Forschungsgemeinschaft for support of this work. We also thank the National Science Foundation for financial support to P. Hedden (GB-41874 Phinney) and D. J. Chapman (GB-42461).

References

1. Shimizu, S. and E. Tamki (1962) Botan. Mag. Tokyo 75, 462-467
2. Ellsworth, R.K. (1971) Photosynthetica 5, 226-232
3. Ellsworth, R.K. (1972) Photosynthetica 6, 276-281

4. Chiba, Y., I. Aiga, M. Idemori, Y. Satoh, K. Matsushita and T. Sasa (1967) Plant Cell Physiol. Tokyo 8, 623-635
5. Wellburn, A.R. (1970) Phytochemistry 9, 2311-2313
6. Steffens, D., I. Blos, S. Schoch and W. Rüdiger (1976) Planta 130, 151-158
7. Granick, S. (1967) Biochemistry of Chloroplasts, T.W. Goodwin, ed. Vol. II, p. 401, Academic Press, New York.
8. Ogawa, T., F. Bovey and K. Shibata (1975) Plant Cell Physiol. Tokyo 16, 199-202
9. Wellburn, A.R. (1976) Biochem. Physiol. Pflanzen 169, 265-271
10. Rüdiger, W., J. Benz, U. Lempert, S. Schoch and D. Steffens (1976) Z. Pflanzenphysiol. 80, 131-143
11. Oster, M.O. and C.A. West (1968) Arch. Biochem. Biophys. 127, 112-123
12. Skilleter, D.N. and R.G.O. Kekwick (1970) Phytochemistry 9, 153-156
13. Watts, R.B. and R.G.O. Kekwick (1974) Arch. Biochem. Biophys. 160, 469-475.