Incorporation of atmospheric oxygen into the carbonyl functionality of the protochlorophyllide isocyclic ring

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Detached cucumber (Cucumis sativus L. var. Beit Alpha) cotyledons incubated in darkness with 5-aminoacetylaminic acid and either 16O2 air (control) or 18O2 in N2 accumulated protochlorophyllide. This was converted into methyl phaeoporphyrin 6a and analysed by mass spectrometry. The molecular ion of the methyl phaeoporphyrin 6a derived from the 18O2 incubation was 2 mass units greater than that of the control, establishing that the oxo oxygen atom of the isocyclic ring is derived from atmospheric oxygen.

INTRODUCTION

MgProto IX monomethyl ester (oxidative) cyclase (cyclase for short) is a complex of enzymes that catalyse the formation of the Chl isocyclic ring. Granick's (1950) hypothetical scheme for this cyclization involved the β-oxidation of the 6-methyl propionate side chain of MgProtoMe to a methyl β-oxopropionate group. In analogy to the β-oxidation of fatty acids, the modification of the 6-methyl propionate side chain was proposed to go through 6-acrylate and 6-β-hydroxypropionate intermediates. Subsequently, these intermediates were detected by Ellsworth & Aronoff (1968, 1969) in extracts from Chlorella mutants.

In our laboratory we have assayed the activity of the cyclase enzyme system both in isolated plastids (Chereskin et al., 1982) and in a reconstituted system obtained from developing cucumber chloroplasts (Wong & Castelfranco, 1985). Absolute requirements for NADPH and O2 were observed (Chereskin et al., 1982; Wong & Castelfranco, 1984), suggesting the involvement of a direct β-hydroxylation step in the formation of the isocyclic ring. Later, we synthesized the intermediates in Granick's scheme and confirmed that the 6-β-hydroxy and 6-β-oxo derivatives of MgProtoMe are substrates for cyclization (Wong & Castelfranco, 1984, 1985; Wong et al., 1985).

Recently we have confirmed and extended the previous findings with synthetic substrates (Walker et al., 1988). The synthetic 6-methyl acrylate proved to be completely inactive as a substrate for cyclization, and only one of the two enantiomeric β-hydroxypropionates was capable of being cyclized. We interpreted these findings in terms of an asymmetric hydroxylation reaction involving atmospheric O2.

In the present study we have tested this hypothesis by incubating detached cucumber cotyledons with 5-aminoacetylaminic acid in the presence of 18O2. The accumulated Pchlide was 2 mass units larger (by mass spectrometry) than the Pchlide accumulated in the control tissue incubated in the presence of 18O2, thereby providing evidence for the incorporation of atmospheric O2 into the newly synthesized Pchlide.

MATERIALS AND METHODS

Materials

CH2Cl2, triethylamine, tetrahydrofuran, hexanes, methanol, acetone, diethyl ether, NaCl and Na2SO4 were purchased from Fisher Scientific. Trifluoroacetic acid, Diazald and Pd/charcoal (10% palladium on activated carbon) were purchased from Aldrich Chemical Co. Celite was obtained from J. T. Baker Chemical Co. Silica Kieselgel 60 PF254 was purchased from E.M. Science. Aq. NH3 was obtained from Malinckrodt. 5-Aminoacetylaminic acid was purchased from Sigma Chemical Co. 18O2 (> 99%) was purchased from MSD Isotopes.

Methods

Plant tissue. Cucumber (Cucumis sativus L. var. Beit Alpha) seeds were germinated in the dark at 30 °C for 5–6 days (Hardy et al., 1970) and then exposed to white light (60–80 μE·m–2·s–1 photosynthetically active radiation at 30 °C) for 20 h. Tissue incubation (adapted from Granick, 1961) was performed as follows. Cotyledons (120 pairs; approx. 10 g of tissue) were blotted dry and coated with 15 ml of incubation medium (25 mM-5-aminoacetylaminic in 10 mM-potassium phosphate buffer, pH 6.8) in a Petri dish (14 cm diameter). Six Petri dishes were placed in a 2.5-litre vacuum desiccator, which was evacuated and refilled with N2 five times. After a final evacuation, the chamber was refilled with air (control) or with 0.5 litre of 18O2 followed by N2 to 0.1 MPa (1 atm) total pressure. The cotyledons were incubated at 25 °C in the dark for 24 h.

Pigment extraction. The initial extractions were performed under a green safe-light to prevent photo-reduction of newly formed Pchlide. Each Petri dish was drained of excess 5-aminoacetylaminic-containing buffer and the cotyledons were ground to a smooth homogenate in 100 ml of ice-cold acetone/0.1 M-NH4 (4:1, v/v) in a Polytron homogenizer at high speed. Solids were sedimented by centrifugation at 12000 g for 10 min; the pellets were re-extracted until no visible pigment remained. All supernatant fractions were pooled.

Abbreviations used: Chl, chlorophyll; Chlide, chlorophyllide; Pchlide, (monovinyl or divinyl) protochlorophyllide; MgProtoMe, magnesium-phosphatephorin monomethyl ester; e.i., electron impact; f.a.b., fast atom bombardment.

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was demetallated extracts Methanolic into the extracted. Then 0.1 vol. of water was added. After 5 min of saturated NaCl.

Also shown is the chemical conversion of Pchlide a (A) into methyl phaeoporphyrin a₅ (D) via 2,4-divinylphaeoporphyrin a₄ (B) and methyl 2,4-divinylphaeoporphyrin a₅ (C). Also shown is methyl mesophaeoporphide a (E). See the text for details.

The acetone extract was washed with 0.5 vol. of hexanes and extracted with diethyl ether by the addition of saturated NaCl. The diethyl ether fraction was diluted with an equal volume of light petroleum (b.p. 60–80 °C). Then 0.1 vol. of methanol/0.01 m-NH₃ (4:1, v/v) was added. The lower green phase was collected, and the procedure was repeated until no further pigments were extracted into the polar lower phase (Griffiths, 1978). Methanolic extracts were pooled and dried in vacuo.

Preparation of methyl phaeoporphyrins. The biosynthetic porphyrin product (compound A, Scheme 1) was demetallated by treatment with 100% (v/v) trifluoroacetic acid (2 ml) for 2–3 min at 22 °C, then diluted with CH₂Cl₂ (290 ml), and washed with water (3 x 50 ml) to remove excess acid. The CH₂Cl₂ phase was dried over Na₂SO₄ and evaporated to dryness. The residue (compound B) was dissolved in methanol (15 ml) and treated with about 20 ml of ethereal diazomethane (prepared in accordance with Fieser & Fieser, 1967). Porphyrin esterification was monitored by t.l.c. on silica-gel plates developed with methanol/CH₂Cl₂ (1:19, v/v). After completion of the diazomethane treatment, the pigments were applied to pre-run silica-gel preparative t.l.c. plates and developed with tetrahydrofuran/CH₂Cl₂ (1:49, v/v). The major porphyrin fraction (compound C) was collected. Porphyrins in 20 ml of tetrahydrofuran were hydrogenated at 22 °C over approx. 10 mg of 10% Pd charcoal. Hydrogenation was continued for a maximum of 2 h, by which time the uptake of H₂ ceased. The Pd charcoal was removed by filtration through Celite. Porphyrin was purified by chromatography on pre-run silica-gel preparative t.l.c. plates developed with tetrahydrofuran/CH₂Cl₂ (1:49, v/v). The major band, methyl phaeoporphyrin a₅ (compound D), was collected and crystallized from CH₂Cl₂/light petroleum.

RESULTS

The major product of the cotyledon incubation with 5-amino-lactate was Pchlide: typically, 60 g of cotyledons yielded 5–10 mg of Pchlide. In cucumbers, the Pchlide formed under these conditions is predominantly in the divinyl form (Belanger & Rebeiz, 1980). To ensure homogeneity in the porphyrin with respect to the 2- and 4-positions and, more specifically, to remove the possibility of erroneous M+2 peaks in the mass spectra (Smith, 1975), the Pchlide was converted into methyl phaeoporphyrin a₅ (compound D, Scheme 1). Removal of magnesium and esterification of the 7-propionic acid group increased the volatility and improved the stability of the compound, decreasing the degradation before and during spectral analysis (Smith, 1975).

During the extraction of Pchlide from cotyledons, variable but small amounts of Chlide were formed as a result of chlorophyllase activity on pre-existing Chl (Holden, 1961). Although Chlide contamination could be avoided by the use of etiolated tissue, it was considered possible that Pchlide synthesis in etiolated tissue might differ from that in the semi-green tissue on which our previous observations were based; therefore we decided to use 20 h-light-exposed tissue in this study as well.

The structure of the methyl phaeoporphyrin a₅ obtained from the Pchlide was confirmed by 1H-n.m.r. spectroscopy. As expected, both the 15O⁻ and the 16O⁻-incubated samples gave identical spectra. Fig. 1 shows the typical 1H-n.m.r. spectrum of the 16O⁻ control. The salient features in the spectrum are the meso hydrogen peaks (a in Fig. 1) at 10.0–10.2 p.p.m., which point to the purity of the isolated porphyrin. The absence of vinyl peaks combined with the presence of two overlapping ethyl CH₂ triplets (d in Fig. 1) also demonstrate complete conversion of the 2- and 4-vinyl groups into ethyl groups.

Spectrophotometry and 1H-n.m.r. spectroscopy indicated less than 5% contamination of the resulting methyl phaeoporphyrin a₅ with methyl mesophaeophorbid e a (compound E, Scheme 1). In the control sample no trace of this phaeophorbid e was detected.

Fig. 2 shows the low-resolution f.a.b. (M+1 ions) mass spectra of both samples. Clearly the molecular ion in the 16O⁻-incubated sample (m/z 609 in Fig. 2b) is 2 mass units greater than in the control (m/z 607 in Fig. 2a). Moreover, the peak in Fig. 2(a) at 547 (characteristic of methoxycarboxyl cleavage) (Smith, 1975) is observed at m/z 549 in Fig. 2(b), indicating 15O content.
Fig. 1. 300 MHz $^1$H-n.m.r. spectrum (GE QE300 instrument) in (D)chloroform of methylphaeoporphyrin $a_1$ synthesized from isolated Phlide $a$, in accordance with Scheme 1 (compounds A–D).

Assignments: $a$, meso ($\alpha$, $\beta$, and $\delta$) protons; $b$, 10-H; $c$, methylene and methyl protons; $d$, terminal methyl groups of the 2- and 4-ethyl groups; $e$, N–H protons.

Fig. 2. F.a.b.-mode ($M+1$) mass spectra of ($a$) control methylphaeoporphyrin $a_1$ (compound D, Scheme 1) derived from a $^{18}$O$_2$ incubation and ($b$) methylphaeoporphyrin $a_1$ derived from the $^{18}$O$_2$ incubation.

These porphyrins were obtained by chemical modification of the biosynthetic product as outlined in Scheme 1. A ZAB-HS-2F mass spectrometer (VG Analytical) was used (8 keV Xe$^+$, 1 mA beam current; dithiothreitol/dithioerythritol matrix).
phaeoporphyrin \( a_5 \) containing one \(^{18}\text{O} \) atom, or (2) the e.i.-mode \( m/z \) 608 peak corresponds to methyl mesophaeoporphide \( a \) containing \(^{18}\text{O} \) atoms. For alternative (1) the measured mass error (Table 1) is 0.4 p.p.m., whereas for alternative (2) the measured mass error would be 18.0 p.p.m., far above the error baseline established for the experiment (Table 1). The only conclusion, therefore, is that the \( m/z \) 609 peak in Fig. 2(b) is derived exclusively from methyl phaeoporphyrin \( a_5 \) containing one non-ester atom of \(^{18}\text{O} \).

### DISCUSSION

In this paper we have studied the fate of the molecular \( \text{O}_2 \) in a Pchlide-\( a \)-synthesizing system by replacing air with an atmosphere of \(^{18}\text{O}_2 \) in \( \text{N}_2 \) during our incubations. In Fig. 2 the molecular ion of the methyl phaeoporphyrin \( a_5 \) derived from the \(^{18}\text{O}_2 \)-incubated sample (609) was clearly 2 mass units greater than that in the control sample (607): again, the same difference of 2 mass units is seen for the fragment at 549 \((^{18}\text{O}_2)\) and 547 (control), which represents loss of a carboxymethyl group, suggesting that this group on the 6- or 7-side chains cannot be the source of the \(^{18}\text{O} \). This is not unexpected, since the carboxy groups on both the 6- and 7-propionic acid side chains of protoporphyrin IX are derived from the carboxyl functionality of the 5-aminolaevulinate used in the incubation (Wriston et al., 1955; Shemin et al., 1955), and this substrate was not \(^{18}\text{O} \)-enriched. We conclude that these data are consistent with the definitive incorporation of one \(^{18}\text{O} \) atom into the carbonyl functionality of the Pchlide isocyclic ring from atmospheric \( \text{O}_2 \).

The isolation of the acrylate derivative of MgProtoMe from extracts of \textit{Chlorella} mutants by Ellsworth & Aronoff (1968, 1969) is not a strong argument against a direct hydroxylation mechanism using molecular \( \text{O}_2 \). The presence of an aromatic ring system on one side of the secondary alcohol, and of a methoxyacarbonyl group on the other side, would certainly activate the C–O bond and facilitate the dehydration of the methyl hydroxypropionate side chain to afford a methyl acrylate side chain. Therefore the acrylate detected by these workers could well be formed from the \( \beta \)-hydroxypropionate intermediate either \textit{in vivo}, during the growth of the mutant, or later, during the course of the isolation.

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### REFERENCES


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