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-aminolaevulinic acid and either $^{16}\text{O}_2$ air (control) or $^{18}\text{O}_2$ in N_2 accumulated protochlorophyllide. This was converted into methyl phaeoporphyrin a_5 and analysed by mass spectrometry. The molecular ion of the methyl phaeoporphyrin a_5 derived from the $^{18}\text{O}_2$ 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 O_2 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 O_2 .

In the present study we have tested this hypothesis by incubating detached cucumber cotyledons with 5-aminolaevulinic acid in the presence of $^{18}\text{O}_2$. The accumulated Pchlde was 2 mass units larger (by mass spectrometry) than the Pchlde accumulated in the control tissue incubated in the presence of $^{16}\text{O}_2$, thereby providing evidence for the incorporation of atmospheric O_2 into the newly synthesized Pchlde.

MATERIALS AND METHODS

Materials

CH_2Cl_2 , triethylamine, tetrahydrofuran, hexanes, methanol, acetone, diethyl ether, NaCl and Na_2SO_4 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. NH_3 was obtained from Malinkrodt. 5-Aminolaevulinic acid was purchased from Sigma Chemical Co. $^{18}\text{O}_2$ (> 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 $\mu\text{E}^{-2}\cdot\text{s}^{-1}$ 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-aminolaevulinate 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 N_2 five times. After a final evacuation, the chamber was refilled with air (control) or with 0.5 litre of $^{18}\text{O}_2$ followed by N_2 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 Pchlde. Each Petri dish was drained of excess 5-aminolaevulinate-containing buffer and the cotyledons were ground to a smooth homogenate in 100 ml of ice-cold acetone/0.1 M- NH_3 (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; Pchlde, (monovinyl or divinyl) protochlorophyllide; MgProtoMe, magnesium-protoporphyrin monomethyl ester; e.i., electron impact; f.a.b., fast atom bombardment.

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Scheme 1. Chemical conversion of Pchlide *a* (A) into methyl phaeoporphyrin *a*₅ (D) via 2,4-divinylphaeoporphyrin *a*₅ (B) and methyl 2,4-divinylphaeoporphyrin *a*₅ (C)

Also shown is methyl mesophaeophorbide *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 × 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 with approx. 200 μl of triethylamine as a catalyst. 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 *a*₅ (compound D), was collected and crystallized from CH₂Cl₂/light petroleum.

RESULTS

The major product of the cotyledon incubation with 5-aminolaevulinic acid 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 *a*₅ (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 *a*₅ obtained from the Pchlide was confirmed by ¹H-n.m.r. spectroscopy. As expected, both the ¹⁶O₂- and the ¹⁸O₂-incubated samples gave identical spectra. Fig. 1 shows the typical ¹H-n.m.r. spectrum of the ¹⁸O₂ 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 CH₃ 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 *a*₅ with methyl mesophaeophorbide *a* (compound E, Scheme 1). In the control sample no trace of this phaeophorbide 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 ¹⁸O₂-incubated sample (*m/z* 609 in Fig. 2*b*) is 2 mass units greater than in the control (*m/z* 607 in Fig. 2*a*). Moreover, the peak in Fig. 2*a*) at *m/z* 547 (characteristic of methoxycarbonyl cleavage) (Smith, 1975) is observed at *m/z* 549 in Fig. 2*b*), indicating ¹⁸O content

Fig. 1. 300 MHz ^1H -n.m.r. spectrum (GE QE300 instrument) in (^2H) chloroform of methyl phaeoporphyrin a_5 synthesized from isolated Pchl a_5 in accordance with Scheme 1 (compounds A-D)

Assignments: *a*, meso (α , β and δ) protons; *b*, 10-H; *c*, methylene and methyl protons; *d*, terminal methyl groups of the 2- and 4-ethyl groups; *e*, N-H protons.

in non-ester functions (i.e. the C-9 C=O). The major expected fragmentation pattern of the 7-propionate group should be loss of 73 mass units (m/z 607 $\rightarrow$ 534; $-\text{CH}_2\text{CO}_2\text{Me}$) (Smith, 1975). However, under the conditions of this particular experiment this peak is not intense enough to enable us to draw any conclusions.

We were concerned, however, that a portion of the peak intensity at m/z 609 in Fig. 2(b) might be the result of the minor amount of contamination by methyl mesophaeophorbide *a* (compound E, Scheme 1), which also has an f.a.b. M_r of 609. Therefore a high-resolution e.i. m.s. study was initiated.

Table 1 shows the calculated and observed high-resolution mass measurements (e.i. mode, M^+) for authentic methyl mesophaeophorbide *a*, and establishes the error for the instrument to be in the 3.5 p.p.m. region. The mass measurements for the authentic control sample of methyl phaeoporphyrin a_5 reveal a comparable 1.5 p.p.m. error. Results from the mass measurement of the material from the $^{18}\text{O}_2$ incubation may be interpreted in two ways: (1) the e.i.-mode m/z 608 peak (equivalent to f.a.b.-mode m/z 609; Fig. 2b) corresponds to methyl

Fig. 2. F.a.b.-mode ($M+1$) mass spectra of (a) control methyl phaeoporphyrin a_5 (compound D, Scheme 1) derived from a $^{16}\text{O}_2$ incubation and (b) methyl phaeoporphyrin a_5 derived from the $^{18}\text{O}_2$ 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^0 , 1 mA beam current; dithiothreitol/dithioerythritol matrix).

Table 1. Calculated and observed high-resolution mass measurements (e.i. mode)

A ZAB-HS-2F mass spectrometer (VG Analytical) was used, with direct insertion probe, 70 eV and source temperature approx. 200 °C.

	Calculated	Observed	Δ (p.p.m.)
Methyl mesophaeophorbide <i>a</i> (C ₃₆ H ₄₀ N ₄ O ₅)	608.299871	608.301994	3.5
Methyl phaeoporphyrin <i>a</i> ₅	606.284221	606.283279	1.6
9-C=16O + 2H	608.299871	608.287537*	20.3
9-C=18O	608.288465	608.287537*	1.5

* Denotes the observed *M_r* of methyl phaeoporphyrin *a*₅ collected from incubation studies in ¹⁸O₂.

phaeoporphyrin *a*₅ containing one ¹⁸O atom, or (2) the e.i.-mode *m/z* 608 peak corresponds to methyl mesophaeophorbide *a* containing ¹⁶O 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 ¹⁸O.

DISCUSSION

In this paper we have studied the fate of the molecular O₂ in a Pchlide *a*-synthesizing system by replacing air with an atmosphere of ¹⁸O₂ in N₂ during our incubations. In Fig. 2 the molecular ion of the methyl phaeoporphyrin *a*₅ derived from the ¹⁸O₂-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 (¹⁸O₂) 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 ¹⁸O. 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 carboxylic functionality of the 5-aminolaevulinate used in the incubation (Wriston *et al.*, 1955; Shemin *et al.*, 1955), and this substrate was not ¹⁸O-enriched. We conclude that these data are consistent with the definitive incorporation of one ¹⁸O atom into the carbonyl functionality of the Pchlide isocyclic ring from atmospheric O₂.

The isolation of the acrylate derivative of MgProtoMe from extracts of *Chlorella* mutants by Ellsworth & Aronoff (1968, 1969) is not a strong argument against a direct hydroxylation mechanism using molecular O₂. The presence of an aromatic ring system on one side of the secondary alcohol, and of a methoxycarbonyl 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 β -hydroxypropionate

intermediate either *in vivo*, during the growth of the mutant, or later, during the course of the isolation.

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