Volume 323, number 1,2, 31–34 FEBS 12467 May 1993
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Derivation of the formyl-group oxygen of chlorophyll *b* from molecular oxygen in greening leaves of a higher plant (*Zea mays*)

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Received 26 March 1993

Using mass spectroscopy, we demonstrate as much as 93% enrichment of the 7-formyl group oxygen of chlorophyll *b* when dark-grown, etiolated maize leaves are greened under white light in the presence of $^{18}O_2$. This suggests that a mono-oxygenase is involved in the oxidation of its methyl group precursor. The concomitant enrichment of about 75% of the 13^1-oxygen confirms the well-documented finding that this oxo group, in both chlorophyll *a* and *b*, also arises from O_2. High ^{18}O enrichment into the 7-formyl oxygen relative to the substrate $^{18}O_2$ was achieved by optimization of the greening conditions in combination with a reductive extraction procedure. It indicates not only a single pathway for Chl *b* formyl group formation, but also unequivocally demonstrates that molecular oxygen is the sole precursor of the 7-formyl oxygen.

Biosynthesis; Photosynthesis; Chlorophyll *b*; ^{18}Oxygen; Isotope exchange; Reductive extraction, Oxygenase

1. INTRODUCTION

Chl *b*, a light-harvesting antenna pigment in the photosynthetic apparatus of higher plants, green algae and prochlorophytes, differs from Chl *a* by possession of a formyl rather than a methyl group at C-7. The enzyme(s) involved in the oxidation of this methyl group to a formyl substituent have not been demonstrated in cell-free preparations, and the reaction mechanism is still unknown; indeed, the substrate of this oxidation is unclear and may be Chl *a*, chlorophyllide *a* or protochlorophyllide (for review see [1,2]).

In this paper, we investigate the proposition that the formation of the formyl group of Chl *b* from a methyl substituent involves a mixed-function oxidase (monooxygenase) in which case the oxygen of the 7-formyl group would arise from molecular oxygen. Etiolated maize leaves were greened in the presence of $^{18}O_2$ and the chlorophylls extracted and examined by mass spectroscopy. $NaBH_4$ was employed in the extraction to convert Chl *b* to [7-hydroxymethyl]-Chl *b* to minimize loss of ^{18}O from the formyl group by reversible *gem*-diol (hydrate) formation with $H_2{}^{16}O$ in the leaf. We demonstrate enrichments of approximately 93% of the 7-formyl oxygen of Chl *b* and of about 75% in the 13^1-oxygen of both Chls *a* and *b*. Recently, and independently, Schneegurt and Beale [3] reported 34% incorporation of label from $^{18}O_2$ into the formyl group of Chl *b* during greening of a mutant of the green alga, *Chlorella vulgaris*: precautions against *gem*-diol formation involved only rapid extraction at low temperature.

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEAE-cellulose, diethylaminoethyl-cellulose; FAB, fast-atom-bombardment; Phe, phaeophytin. The IUB-IUPAC approved numbering system for tetrapyrroles has been used with bracket [] nomenclature for substitutions. [7-hydroxymethyl]-Chl *b* is identical to [7-hydroxymethyl]-Chl *a* but will be referred to here as a Chl *b* derivative to indicate its origin.

2. EXPERIMENTAL

2.1. *Chemicals*

$^{18}O_2$ with 99.5% isotope enrichment was supplied by Novachem Pty. Ltd., Melbourne, Australia. DEAE-cellulose (DE 52), supplied by Whatman Laboratory Division, Maidstone, England, was treated and suspended in methanol [4]; the DEAE-cellulose was then equilibrated with $CHCl_3$.

2.2. *Organisms, growth and greening conditions*

Three etiolated leaves, excised from 20-day-old, dark-grown maize seedlings (*Zea mays* hybrid var. Dekalb XL689) [5], were placed into each of six Thunberg tubes (50 ml capacity) containing 2 ml of deoxygenated water and filled with $^{18}O_2$ (approx. 70%) and O_2-free N_2 (approx. 30%) by standard vacuum-line techniques. The leaves in the sealed tubes were then greened by illumination with white light (50–60 $\mu E\ m^{-2} \cdot s^{-1}$) for up to 26 h at 27°C (cf. [6])

2.3. *Extraction and purification of chlorophylls from greened maize leaves*

Leaves were extracted by grinding in a chilled mortar in a freshly prepared solution containing equimolar (16.5 mM) $NaBH_4$ and 8-hydroxyquinoline. After centrifugation a clear methanolic supernatant containing Chl *a* and [7-hydroxymethyl]-Chl *b* with few chlorophyll by-products was obtained. The chlorophylls were transferred to diethylether before evaporating to dryness under reduced pressure.

Table I

Theoretical intensity distribution of the clusters derived from the natural abundances of the C, H, N, O, and Mg isotopes

		$(M)^+$	$(M + 1)^+$	$(M + 2)^+$	$(M + 3)^+$	$(M + 4)^+$
Chl *a*	$C_{55}H_{72}N_4O_5Mg$	100.0	77.2	43.8	16.7	4.4
Phe *a*	$C_{55}H_{74}N_4O_5$	100.0	64 3	21.4	4.8	0.8
[7-CH_2OH]-Chl *b*	$C_{55}H_{72}N_4O_6Mg$	100.0	72.2	44.0	16.9	4.5

The two major chlorophylls in the residue were separated on a column of $CHCl_3$-equilibrated DEAE-cellulose developed with $CHCl_3$. The eluant was monitored between 680–630 nm and 480–400 nm with a Shimadzu UV1202 spectrophotometer: the carotenoids were eluted first followed by Chl *a* absorbing at 666 and 432 nm. When [7-hydroxymethyl]-Chl *b*, absorbing at 659 and 434 nm, began to elute, 2% CH_3OH was added to the solvent to elute this derivative rapidly.

2.4. *Mass spectroscopy*

The Chl *a* and [7-hydroxymethyl]-Chl *b* were dissolved in methylene chloride and mass spectra recorded using liquid surface ionization technique in a *m*-nitrobenzyl-alcohol matrix using a model MAT9000 mass spectrometer (Finnigan-MAT, Bremen) with a Caesium gun (20 kV, ≈ 3 μA) and 1200 mass resolution After a survey in the exponential scan mode, 20–25 spectra of the molecular ion region in the linear scan mode were averaged The ^{18}O-isotope enrichment was calculated on the basis of the theoretical intensity distribution of the clusters derived from the natural abundances of the C, H, N, O and Mg isotopes (Table I).

Furthermore, adjustment was made for the varying amounts of M^+ and MH^+ ions arising from porphyrins and chlorins [7].

3. RESULTS

Extraction of leaves after greening with equimolar $NaBH_4$ and 8-hydroxyquinoline in methanol followed by DEAE-cellulose chromatography yielded Chl *a* and [7-hydroxymethyl]-Chl *b* (see section 2): the 13^1-oxo group was not reduced under these conditions. FAB-mass spectroscopy of the Chl *a* showed one ^{18}O label per tetrapyrrole molecule when the excised leaves were greened in an $^{18}O_2$-containing atmosphere (Table II). The isotope enrichments were 75 and 72% after 16 and 26 h, respectively. Since Chl *a* is partially demetalated under FAB conditions, the Phe *a* molecular ion was analysed in parallel: the slightly higher incorporation values (77 and 76%) allow an estimate of the precision of the mass spectra analyses. This ^{18}O label is incorporated into the 13^1-oxo group of Chl *a* by an oxygenase [8] during the obligatorily aerobic process of *iso*cyclic ring formation [9].

The Chl *b* derivative contained two ^{18}O labels per tetrapyrrole molecule after incubation of leaves in the $^{18}O_2$-containing atmosphere: the first is again located in the 13^1-oxo group and the second, therefore, must be the 7-formyl oxygen. When calculating 7-formyl ^{18}O labelling, it was assumed that labelling at the 13^1-oxo group of Chl *b* was the same as in Chl *a*. With this assumption, the labelling of the 7-formyl oxygen was 93 and 92% after 16 and 26 h incubations, respectively. This is close to the isotopic purity of the precursor $^{18}O_2$ (see section 2) and has important implications (see section 4.1).

In the experiments reported, the photosynthesis inhibitor DCMU was not used to prevent dilution of $^{18}O_2$ by inhibiting $^{16}O_2$ production from $H_2{}^{16}O$ in the cell sap. A comparison of the isotope enrichment after 16 and 26 h showed a decrease of 1–4% over this period of 10 h indicating minor dilution by photosynthetically pro-

Table II

^{18}O-labeling of chlorophylls during greening

Pigment	Greening time (h)	Molecular ion intensities (%)			Labeling[d] (%)	
		^{16}O	^{18}O	$^{18}O_2$	$^{18}O_A$	$^{18}O_B$
Chl *a*		892.5 m/z	894.5 m/z			
	16	32	100	–	75	–
	26	39	100	–	72	–
Phe *a*		870.5 m/z	872.5 m/z			
	16	30	100	–	77	–
	26	31	100	–	76	–
[7-Hydroxy-methyl]-Chl *b*[a]		908 5 m/z	910 5 m/z	912.5 m/z		
	16	10	44	100	76[b,c]	93
	26	14	55	100	74[b c]	92

[a][7-hydroxymethyl]-Chl *b* is only little demetalated under the measuring conditions, therefore the [7-hydroxymethyl]-Phe *b*-derived ions could not be used for determining the $^{18}O_2$ enrichment. [b]Median of the labeling of Chl *a*/Phe *a* at the respective times. See text [c]According to the mass spectral analysis, the sample contains minor amounts of [3-ethyl]-[7-hydroxymethyl]-chlorophyll *b*. [d]Subscripts A and B refer to two independently labeled sites. The similar enrichment of site A in Chl *a*, Phe *a* and the Chl *b*-derivative was the basis for assigning it to the 13^1-oxygen, then site B is the 7^1-oxygen.

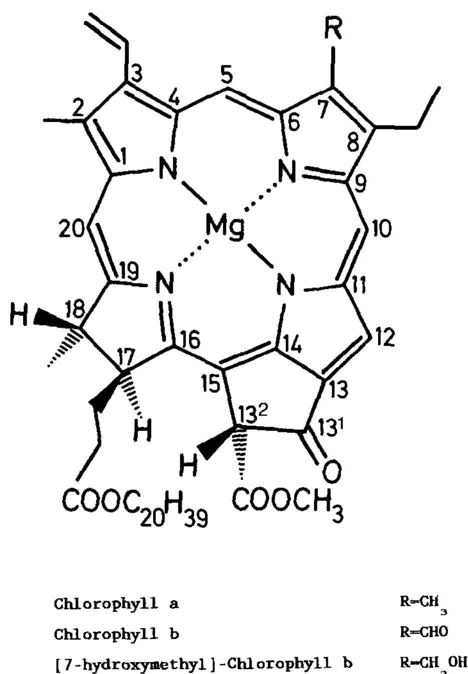

Fig. 1.

duced $^{16}O_2$. Assuming rapid equilibration of the photosynthetically produced $^{16}O_2$ with the $^{18}O_2$, this 1–4% decrease in isotope enrichment corresponds to production of about 0.5–2.0 ml of $^{16}O_2$ per 50 ml Thunberg tube over the 10 h period. It has been shown that C_4 plants, when photosynthesizing maximally, evolve 0.07 μmol of O_2 per μE absorbed [10]. Thus three leaves with a total area of 25 cm^2 in a Thunberg tube would produce 8.5 ml of $^{16}O_2$ in 10 h at 60 $\mu E \cdot m^{-2} \cdot s^{-1}$. However, after 26 h in the light the leaves are not fully greened and there is considerable self shading in the Thunberg tube. Also, there is a significant lag in the development of O_2-evolving activity in greening leaves [11]. Thus formation of 0.5–2.0 ml of $^{16}O_2$ over 10 h in this experiment is not unreasonable.

4. DISCUSSION

In designing the experiment several critical points were considered including the greening conditions. Earlier experiments conducted in closed Thunberg tubes showed that pure $^{16}O_2$ inhibited greening by about 50% relative to greening in open tubes under normal atmospheric conditions. Because the $^{18}O_2$ studies were also done in closed Thunberg tubes, an atmosphere of approximately 70% $^{18}O_2$ and 30% N_2 was chosen since there was little or no inhibition of chlorophyll biosynthesis under these conditions.

Briefly mentioned already, another experimental design consideration was the possible exchange of the formyl oxygen by reversible *gem*-diol formation. Assuming no isotopic effect, this would dilute the label 50% for every turnover. To overcome this problem, the 7-formyl was reduced to a 7-hydroxymethyl-group with $NaBH_4$ in methanol during extraction. This reduction, however, imposed the very considerable experimental difficulty of finding a method to inhibit the concomitant reduction of the 3-vinyl group of Chl *b*, which unexpectedly occurred in crude methanol extracts of leaves. We found that this reduction, which produces [3-ethyl]-[7-hydroxymethyl]-Chl *b*, is inhibited by 7-hydroxyquinoline: this is the subject of a separate publication. The higher ^{18}O enrichment of the 7-formyl group relative to the 13^1-oxo group indicates that this isotope loss is efficiently inhibited by this reductive extraction procedure. The high incorporation of isotope from $^{18}O_2$ into the formyl group of Chl *b* also suggests that reversible *gem*-diol formation during the long 26 h greening period in the presence of $H_2{}^{16}O$ in the cell sap is slow in the hydrophobic environment of the thylakoid membranes.

Recent work with a *Chlorella vulgaris* mutant [3], showed comparable ^{18}O enrichment of the 13^1-oxo group of Chl *a* but enrichment of the 7-formyl-group oxygen was approximately half of that demonstrated here. This indicates that rapid extraction of Chl *b* at low temperature, which was the only precaution against this isotope exchange used in the algal study, is less effective than the reductive extraction procedure discussed above.

4.1. *Concluding remarks*

In summary, it has been shown that the 7-formyl oxygen of Chl *b* is derived from molecular oxygen and that ^{18}O-labelling in excess of 90% can be achieved by careful optimization of the greening conditions and the extraction procedure. The enrichment achieved is close to the isotopic purity of 99.5% of the precursor $^{18}O_2$ (see section 2). This very small isotopic dilution suggests a single oxygenase pathway for formyl group formation and unequivocally demonstrates that molecular oxygen is the sole precursor of the formyl oxygen. Since monooxygenases generally yield alcohol as products, either a single enzyme [12] with unusual properties leading directly to a carbonyl group is required or an additional dehydrogenase.

Acknowledgements: One of us (R.J.P.) thanks the Deutscher Akademischer Austauschdienst (DAAD) for financial support during his visit to the Botanisches Institut der Universität, München, to complete this work. This work was also in part supported by the Deutsche Forschungsgemeinschaft (SFB 143, Projekt A9) and the CSIRO-Division of Plant Industry, Canberra, Australia.

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