Evidence for a monooxygenase-catalyzed primary process in the catabolism of chlorophyll

Christophe Curty, Norbert Engel, Albert Gossauer*

Institut für Organische Chemie der Universität, ch. du Musée 9, Pérolles, CH-1700 Fribourg, Switzerland

Received 16 March 1995

Abstract **Only recently have products of the enzymatic breakdown of the chlorophylls been characterized for the first time. All catabolites isolated until now from a chlorophyte and from angiosperms have in common the position at which the primary oxygenative ring cleavage occurs, yielding linear tetrapyrroles (19-formyl-1[21H,22H]bilinones). In vivo studies of $^{18,18}O_2$ incorporation in one of the chlorophyll catabolites isolated from *Chlorella protothecoides* show unequivocally that of the two oxygen atoms inserted into the pigment, only the formyl oxygen originates from dioxygen whereas the other one, the lactamic oxygen atom, derives from water. These findings suggest a monooxygenase-catalyzed primary process in the catabolism of chlorophyll.**

Key words: Chlorophyll catabolism; Monooxygenase; ^{18}O labeling; *Chlorella protothecoides*; 19-Formyl-1[21H,22H]bilinone

1. Introduction

In contrast to the biosynthesis of the chlorophylls, which has been elucidated in great detail in the past decades, virtually nothing was known until recently about the chemical structures and the enzymes involved in the degradation of these essential pigments of live [1]. The global chlorophyll turnover which is estimated at about 10^9 *t* annually is particularly conspicuous in its spectacular degradation during autumnal senescence of leaves.

During the last four years two different research groups have succeeded independently from each other in the structure elucidation of different catabolites of the chlorophylls isolated from such diverse phyla as the chlorophyte *Chlorella protothecoides* [2,3], and from angiosperms like the monocot *Hordeum vulgare* vc. Gerbel (barley, [4]), and the dicot *Brassica napus* L. (rape, [5]). It was surprising to notice that both in algae and higher plants the chromophore of the photosynthetic pigments is regioselectively cleaved by oxygenation at the C4-C5 bond of the chlorophyll macrocycle yielding bile-pigment-like products which resemble the parent chlorophylls by the presence of the characteristic condensed cyclopentane ring (cf. **5** in Fig. 1). In contrast to heme catabolism in which the *meso* carbon atom C5 is released as carbon monoxide during ring cleavage, chlorophyll catabolites retain this atom as a formyl group attached to ring B. It has been anticipated that oxygenolysis of the macrocycle may occur by the action of a dioxygenase, which recognizes a dephytylated form of chlorophyll, presumably pheophorbide [6]. The elucidation of the mechanism by which this unusual enzymatic oxidative ring opening occurs is the objective of the present investigation.

2. Materials and methods

2.1. Chemicals

All commercially available chemicals were reagent grade; solvents were distilled prior to use. Thin layer chromatography (TLC) aluminium foils precoated with silica gel 60 F_{254} (0.2 mm) and preparative TLC plates coated with silica gel 60 $PF_{254+366}$ (1.25 mm thick, 20 × 20 cm) were purchased from E. Merck (D-6100 Darmstadt, Germany). Oxygen mixtures were prepared from natural oxygen taken from an ordinary gas cylinder and from isotopic oxygen, consisting of a mixture of 98.505 atom% ^{18}O, 0.063 atom% ^{17}O, and 1.432 atom% ^{16}O, contained in a break seal flask purchased from Cambridge Isotope Laboratories Inc., Andover, MA 01810-5413, USA.

2.2. Mass spectrometry

Mass spectra were obtained with a Vacuum Generator Micromass 70 70E instrument equipped with a DS 11-250 data system from VG Micromas Ltd. (Manchester, UK) using the fast atom bombardement (FAB) ionization technique in the positive mode with Xe as primary atom beam at 7 keV and 1 μA. Samples were first dissolved in MeOH and then added to the glycerol/1-thioglycerol (1:1) matrix.

2.3. In vivo incubation procedure

In each experiment, 100 ml cultures of *Chlorella protothecoides* (5×10^7 cells/ml) have been grown heterotrophically on 5% glucose in darkness as previously described [3]: air was displaced by nitrogen in an air-tight shaking vessel containing the algae culture. The device was immediately connected through a silicon tubing to a graduated reservoir containing ^{18}O-enriched oxygen maintained under atmospheric pressure. Thus, one run was carried out with $^{16,16}O_2 : {}^{18,18}O_2 = 55.5 : 44.5$ and another with $^{16,16}O_2 : {}^{18,18}O_2 = 11 : 89$. At the beginning of the experiment, an incubation atmosphere of about 80% nitrogen and 20% oxygen was established by suction of oxygen from the reservoir, draining off a calculated amount of aqueous 4 N NaOH solution contained in a compartment of the reactor. Continuous oxygen flow into the reactor was driven by absorption of the carbon dioxide, liberated during glucose consumption, in the NaOH solution mentioned above. Bleaching was interrupted after 48 h during which 250 ml oxygen was consumed. The main chlorophyll *a* catabolite **5** was isolated, purified and analyzed by mass spectroscopy as described before [2,7].

3. Results

In a previous work [7] we demonstrated that photooxygenative ring opening of the chlorophyll *a* derivative, pyropheophorbidato *a* methyl ester Cd(II), in non-aqueous solutions occurs by the so-called 'one molecule mechanism', i.e. both terminal oxygen atoms derive from the same oxygen molecule suggesting that singlet oxygen is involved in the formation of a dioxetane derivative as intermediate. The resulting product resembles the natural chlorophyll catabolites.

In order to gain an insight into the reaction mechanism of the primary enzymatic reaction in chlorophyll catabolism, the origin of the terminal oxygen atoms present in the isolated products is of predominant interest. As a matter of fact, the

*Corresponding author. Fax: (41) (37) 29-9739.

SSDI 0014-5793(95)00348-7

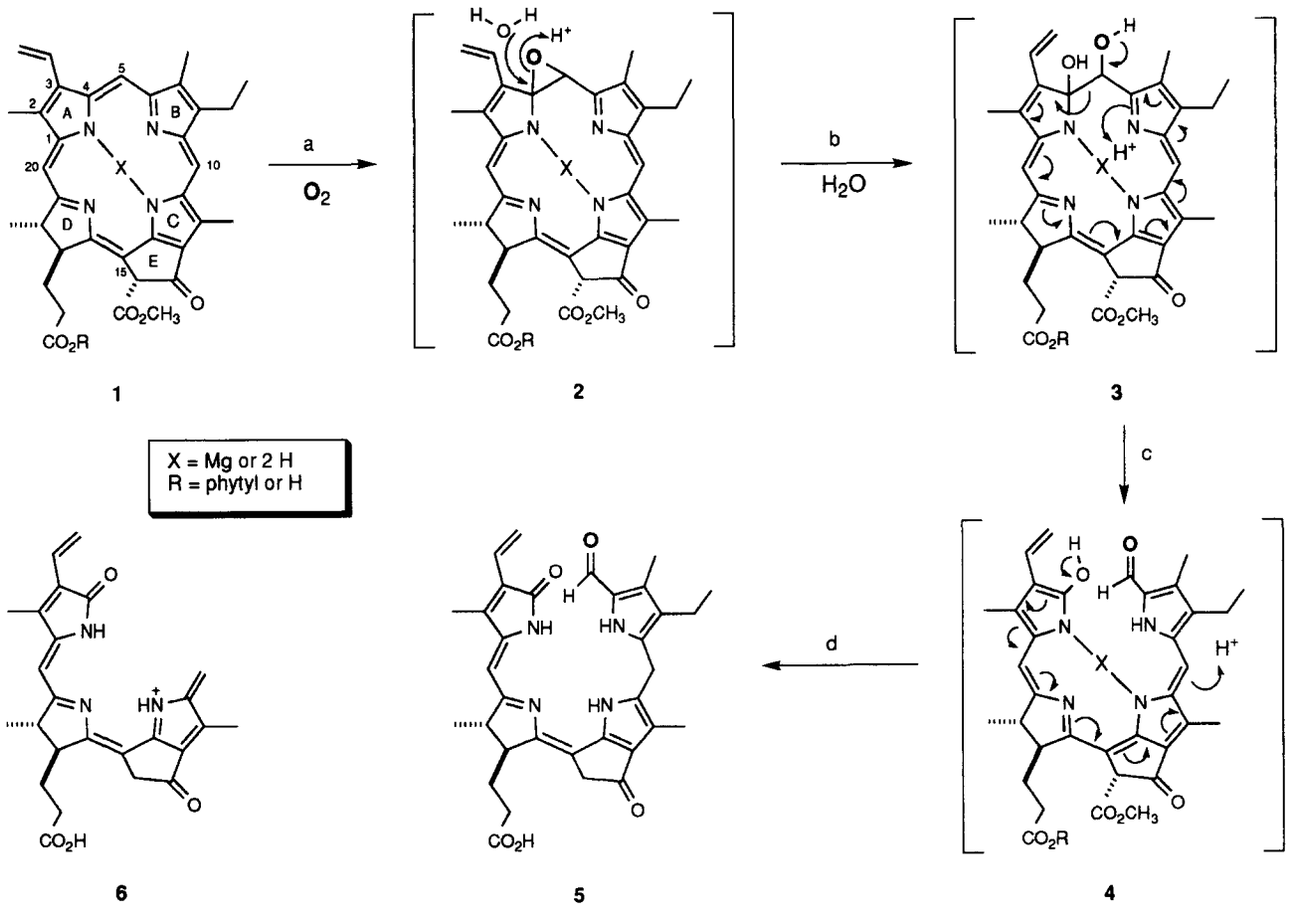

Fig. 1. Mechanism proposed for the ring cleavage of chlorphyll *a* involving the action of a monooxygenase in the key step. The following sequence for the ring opening is suggested: (a) addition of a monooxygenase-activated oxygen molecule to the C4–C5 double-bond yields the oxirane derivative 2; (b) regioselective cleavage of the oxirane ring by spontaneous or enzymatic (epoxyde hydrase) hydrolysis results in the formation of intermediate 3, in which the oxygen label remains bound at position C5; (c) a *retro*-aldol condensation type reaction affords 4; (d) prototropic rearrangement of the latter and subsequent decarbomethoxylation on C13[2] eventually yields the isolated chlorophyll *a* catabolite **5**. Included in the figure is the structure of the mass spectroscopic fragment ion (**6**) at m/z 432, in which the lack of ^{18}O label supports the proposed mechanism.

green algae *C. protothecoides* offers essential advantages compared with pluricelullar organisms for in vivo studies, namely because: (i) the structures of the catabolites isolated from angiosperms are more complex in so far as they contain oxygen atoms on peripheral substituents in addition to the two oxygen atoms concerned with the ring cleavage reaction; and (ii) handling of submerse cultures simplifies the design of the incubation device.

The methodology followed in the present work parallels that which was developed originally by Brown et al. [8] to elucidate the mechanism of heme degradation in vivo. Thus, in order to differentiate between the four conceivable reaction mechanisms (Table 1) batches of living green cells of *C. protothecoides* were incubated with specific isotopic mixtures of $^{18,18}O_2$ and $^{16,16}O_2$ during the process of heterotrophic growth (bleaching) in which the chlorophylls *a* and *b* are catabolized. The isotopic composition of the main chlorophyll *a* catabolite excreted into the culture medium during the process of bleaching was analyzed, after chromatographic separation, by FAB mass spectroscopy as previously described [7].

Although under these conditions the main catabolite is difficult to separate from the accompanying pheophorbide *a* analogue as well as from the corresponding chlorophyll *b* catabolites [3], the mixture of the four pigments was directly used for FAB mass spectrometric analysis, since it contains more than 90% of the chlorophyll *a* catabolite **5**, which has the lowest molecular mass of all components. Actually, the closest contaminant is at least +14 mass units apart from the center of the molecular ion, and as such does not interfere with the mass spectroscopic measurements. FAB mass spectra obtained from the incubation samples were statistically analyzed and are juxtaposed in Fig. 2. The resulting data have been mathematically transformed into the relative amount of pigment labeled with none, one or two ^{18}O atoms (Table 1), as formerly developed [7].

The comparison of the experimentally observed labeling pattern of the pigment with the predicted incorporation rates matches a 'monohydrolytic cleavage mechanism'. Nevertheless, this assignment could be delusive taking into account the possibility of a hydrolytic exchange of one of the terminal oxygen atoms in pigment **5** after a ring cleavage which may take place by a dioxygenation mechanism involving either one or two oxygen molecules.

It was found, however, that both ^{18}O atoms remain in a double labeled pigment, which was synthesized by photooxygenation (vide supra), when the latter was incubated in the bleach-

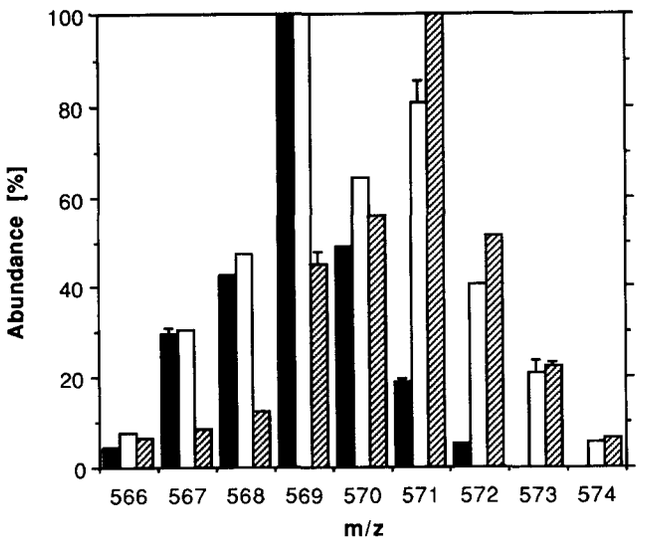

Fig. 2. Juxtaposed intensity patterns in the range of the molecular ion peaks of the FAB spectra of **5**, isolated from the culture media of *Chlorella protothecoides* using: natural O_2 (black bars); $^{16,16}O_2:{}^{18,18}O_2 = 55.5:44.5$ (white bars); and $^{16,16}O_2:{}^{18,18}O_2 = 11:89$ (hatched bars). Each intensity value represents the mean of ten repeated-scan FAB mass spectra, standard deviations of relevant signals are indiavedd on the top of the bars.

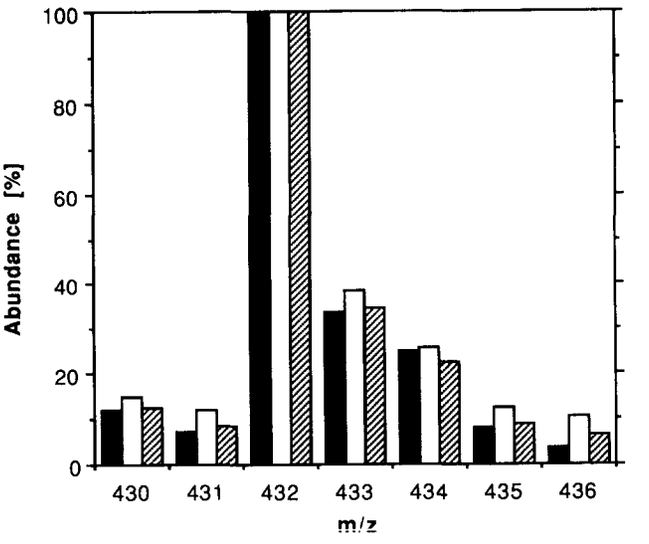

Fig. 3. Juxtaposed intensity patterns in the range of the peaks associated with the fragment ion **6** in the FAB spectra of **5**, isolated from the culture media of *Chrlorella protothecoides*, using: natural O_2 (black bars); $^{16,16}O_2:{}^{18,18}O_2 = 55.5:44.5$ (white bars); and $^{16,16}O_2:{}^{18,18}O_2 = 11:89$ (hatched bars).

ing medium at pH 6.8. In aqueous KOH or HCl solutions, on the contrary, only one oxygen atom is readily exchanged. Mass spectroscopic investigation of the product after hydrolysis shows that the oxygen label remains in fragment ion **6** (actually the basis peak), carrying the lactam group [7]. Therefore, hydrolytic exchange of the oxygen atom must have occurred, as expected, at the formyl group and not at the lactam group.

On the contrary, mass spectrometric analysis of the products obtained from incubation experiments in vivo reveals the lack of label in the fragment ion **6** in all samples investigated, thus proving that the ^{18}O atom must be located in the formyl group (Fig. 3). The exclusive detection of ^{18}O in the formyl group of **5** rules out the possibility of a dioxygenation mechanism involving one or two oxygen molecules with subsequent hydrolytic label exchange and suggests a primary process in the catabolism of chlorophyll which is catalyzed by a monooxygenase.

4. Discussion

The reaction sequence outlined in Fig. 1 is suggested on a chemical plausible basis. Both epoxide formation and hydrolytic epoxide ring cleavage are well-documented enzymatic processes [9]. As epoxide **2** and the final catabolite **5** are on the *same* oxidation level, addition of water is sufficient for the transformation of the former into the latter. This is in contrast to the related oxidative ring opening of heme by heme oxygenase, in which water is not involved in the scission step.

Actually, the specific substrate of the monooxygenase is still unknown. However, as pheophytin *a* is less prone to oxidation than chlorophyll *a* [10], the latter may be favored as a substrate for the enzyme. It also remains unclear at what point in the catabolic process the hydrolysis of the phytyl ester takes place. On the generally accepted assumption that chlorophytes are the phylogenetic ancestors of higher plants [11], the chlorophyll

Table 1
Calculated statistical incorporation of ^{18}O into formylbilinone **5** as predicted for the four possible cleavage mechanisms compared with the experimentally observed data

	$^{16,16}O_2:{}^{18,18}O_2 = 55.5:44.5$			$^{16,16}O_2:{}^{18,18}O_2 = 11:89$		
	m/z			m/z		
Reaction mechanism	569	571	573	569	571	573
One oxygen molecule	55.5	0	44.5	11	0	89
Two oxygen molecules	30.8	49.4	19.8	1.2	19.6	72.2
Mono hydrolytic	55.5	44.5	0	11	89	0
Double hydrolytic	100	0	0	100	0	0
Experimentally observed	53.3 ± 2.2	40.8 ± 5.3	5.9 ± 3.2	14.9 ± 4.1	81.4 ± 2.1	3.7 ± 1.1

In the 'double hydrolytic mechanism' both terminal oxygen atoms in the pigment are derived from water, whereas in the 'mono-hydrolytic mechanism' one atom proceeds from dioxygen and the other from water. 'One oxygen molecule mechanism' means that both atoms are derived from a single dioxygen molecule, whereas in the 'two oxygen molecules mechanism' they originate from two different dioxygen molecules [8]. Thus, the values account for the relative amounts of each molecular species in the sample containing none (m/z = 569), one (m/z = 571) and two (m/z = 573) ^{18}O atoms. In the calculation, small amounts of the mixed dioxygen species $^{18,16}O_2$ present in the gas mixtures have been neglected for simplicity, the resulting error is within the experimental accuracy. Experimentally observed data were calculated from the values shown in Fig. 2 according to our previous work [7] and are presented as mean ± S.E.M.

cleavage mechanism suggested in this work should be common to all photosynthetic eukaryotes.

Acknowledgements: Financial support have been provided by the Swiss National Science Foundation (Project No. 20-36559.92). Mass spectroscopic measurements were carried out by F. Nydegger.

References

[1] Brown, S.B., Houghton, J.D. and Hendry, G.A.F. (1991) in: Chlorophylls (Scheer H. ed.) pp. 465–489, CRC Press, Boca Raton, FL.
[2] Engel, N., Jenny, T.A., Mooser, V. and Gossauer, A. (1991) FEBS Lett. 293, 131–133.
[3] Iturraspe, J., Engel, N. and Gossauer, A. (1994) Phytochemistry 35, 1387–1390.
[4] Kräutler, B.; Jaun, B., Bortlik, K., Schellenberg, M. and Matile, P. (1991) Angew. Chem. 103, 1354–1357; Angew. Chem. Int. Ed. Engl. 30, 1315–1318.
[5] Mühlecker, W., Kräutler, B., Ginsburg, S. and Matile, P. (1993) Helv. Chim. Acta 76, 2976–2980.
[6] Ginsberg, S., Schellenberg, M. and Matile, P. (1994) Plant Physiol. 105, 545–554.
[7] Curty, C., Engel, N., Iturraspe, J. and Gossauer, A. (1995) Photochem. Photobiol. 61 (in press).
[8] Brown, S.B. and King, R.F.G.J. (1978) Biochem. J. 170, 297–311.
[9] de Bont, J.A.M. (1993) Tetrahedron Assymetry 4, 133–1340.
[10] Watanabe, T. and Kobayashi, M. (1991) in: Chlorophylls, (Scheer, H. ed.) pp. 287–315, CRC Press, Boca Raton, FL.
[11] van den Hook, C., Jahns, H.M. and Mann, D.G. (1993) Algen, 3rd edn., chap. 30, Thieme-Verlag, Stuttgart/New York.