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Cleavage of Chlorophyll-Porphyrin[1]

Requirement for Reduced Ferredoxin and Oxygen

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The chemical structures of some colorless catabolites that accumulate in senescent leaves have been established recently (B. Kräutler, B. Jaun, W. Amrein, K. Bortlik, M. Schellenberg, P. Matile [1992] Plant Physiol Biochem 30: 333–346; W. Mühlecker, B. Kräutler, S. Ginsburg, P. Matile [1993] Helv Chim Acta 76: 2976–2980). Such studies suggest that oxygenolytic cleavage of chlorophyll-porphyrin may occur by the action of a dioxygenase. We have attempted to demonstrate such an enzyme activity and to explore the requirements of the cleavage reaction in a reconstituted system of chloroplast (Chlpl) components prepared from senescent rape (*Brassica napus* L.) cotyledons. Intact senescent Chpls (also referred to as gerontoplasts) contain small amounts of two fluorescent chlorophyll catabolites, Bn-FCC-1 and Bn-FCC-2, probably representing primary cleavage products. Upon the incubation of Gpls in the presence of glucose-6-phosphate (Glc6P) or ATP, these catabolites (predominantly FCC-1) were produced in organello. In a reconstituted system of thylakoids and stroma fraction the FCCs (predominantly FCC-2) were produced in the presence of ferredoxin (Fd) and cofactors (NADPH, Glc6P) helping to keep Fd in the reduced state. Reduced Fd could not be replaced by other electron donors, suggesting that the putative dioxygenase requires Fd for the operation of its redox cycle. Production of FCC-2 did not occur in the absence of oxygen and it was inhibited by chelators of Fe^{2+}. The contributions to the production of FCCs from both parts of the reconstituted system, thylakoids and stroma, are heat labile. The enzymic process in the thylakoids yields pheophorbide *a*, the presumptive precursor of FCCs. However, native senescent thylakoids could not be replaced as a "substrate" by free pheophorbide *a*. The stromal enzyme appears to have an affinity for senescent thylakoids; thus, "loaded" thylakoids capable of FCC production in the presence of Fd and cofactors were obtained upon homogenization of senescent cotyledons in a medium containing sorbitol and ascorbate. Such thylakoids were inactive if prepared from mature green cotyledons. As senescence was induced, the capacity to generate FCCs appeared and peaked when about half of the chlorophyll had disappeared from the cotyledons. The effectiveness of a relevant inhibitor showed that cytoplasmic protein synthesis was required for inducing the catabolic machinery in the loaded thylakoids. Thylakoids from mature Chlpls were ineffective as substrate of the stromal enzyme prepared from Gpls. However, senescent thylakoids yielded FCCs if challenged with stroma from either Chlpls or Gpls. Therefore, the stromal part of the system is likely to be a constitutive enzyme, and the pace-setting step of the pathway of chlorophyll breakdown seems to be located in the thylakoids.

The biochemical mechanism responsible for the cleavage of Chl-porphyrin in senescent leaves is still largely unknown. A number of catalytic systems capable of bleaching Chl in vitro have been described (Holden, 1965; Sakai-Imamura, 1975; Matile, 1980; Hougen et al., 1982; Huff, 1982; Martinoia et al., 1982; Lüthy et al., 1984, 1986a, 1986b; Kato and Shimizu, 1985, 1987; Yamauchi and Minamide, 1985; Whyte and Castelfranco, 1993). However, none of these systems has been demonstrated to be relevant for breakdown in vivo. Above all, in none of these cases have cleavage products been characterized and identified as naturally occurring catabolites.

Admittedly, catabolites of Chl-porphyrin accumulated during foliar senescence have only recently been discovered, e.g. by means of radiolabeling of pyrrole units in greening leaves and subsequent tracing of radioactivity in the course of yellowing (Peisker et al., 1990; Ginsburg and Matile, 1993). Catabolites have been purified from *Hordeum vulgare* (Kräutler et al., 1991, 1992), *Chlorella protothecoides* (Engel et al., 1991), and *Brassica napus* (Mühlecker et al., 1993) and identified as linear tetrapyrroles derived from pheophorbide *a*. These compounds have in common that cleavage of the chlorine macrocycle occurred in α (C-4/C-5)-mesoposition without loss of the methene bridge carbon unit. Thus, the oxygenolytic cleavage reaction results in the formation of a formyl residue attached to pyrrole B and a lactam carbonyl in pyrrole A. It appears to be initiated by the addition of molecular oxygen to the double bond of the methene bridge (Fig. 1). Such a reaction is likely to be catalyzed by an enzyme that recognizes a dephytylated form of Chl, presumably pheophorbide, in thylakoids of senescing Chpls. Tentatively, we will use "dioxygenase" as an operational term for this presumptive key enzyme in the catabolic pathway of Chl.

In senescent leaves some catabolites occur in rather large quantities. They belong to a group of colorless NCCs (see Ginsburg and Matile, 1993, for a newly proposed terminol-

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Abbreviations: Chlpl, chloroplast; FCC, fluorescent chlorophyll catabolite; Glc6P, glucose-6-phosphate; Glc6P-DH, glucose-6-phosphate dehydrogenase; Gpl, gerontoplast = senescent Chlpl; MDMP, 2-(4-methyl-2,6-dinitroanilino)-*N*-methyl-propionamide; NCC, nonfluorescent chlorophyll catabolite.

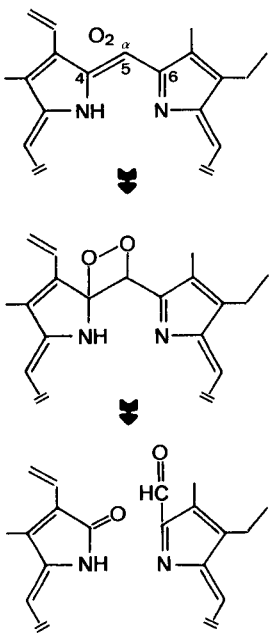

Figure 1. Presumptive mechanism of oxygenolytic cleavage of Chl-porphyrin as inferred from the structure of the natural catabolites Hv-NCC-[RP14] (Kräutler et al., 1991, 1992) and Bn-NCC-1 (Mühlecker et al., 1993).

ogy) representing secondary or final breakdown products that, in the senescent barley mesophyll, have been localized in the vacuoles (Matile et al., 1988; Bortlik et al., 1990). Primary or early catabolites are distinguished by their blue fluorescence (FCCs), minute quantities of which occur in senescent Chlpls when rates of Chl losses in the leaves are high (Düggelin et al., 1988; Schellenberg et al., 1990; Ginsburg and Matile, 1993). In barley, one of the FCCs, Hv-FCC-2, is produced in organello if isolated Gpls[2] are supplied with either ATP (Schellenberg et al., 1990) or Glc6P (Matile et al., 1992). ATP is also required for the release of this FCC from Gpls into the medium, i.e. into the cytosol.

The tracking down of the putative dioxygenase and the analysis of conditions required for the enzymic cleavage of Chl-porphyrin would certainly be impossible unless the reaction yielding a relevant catabolite could be studied in vitro. Attempts to establish a system consisting of components from barley Gpls have yielded preliminary evidence that the reaction requires oxygen and reduced Fd (Schellenberg et al., 1993). However, the unambiguous identification of the FCC produced with a derivative of Chl-porphyrin has failed because of the minuteness of the pools accumulated during incubations. As an alternative, we employed cotyledons of rape seedlings as a source of Chlpls and Gpls. This was a fortunate choice not only with regard to the identification of catabolites (Ginsburg and Matile, 1993), but also with regard to the study of the cleavage reaction.

[2] Senescent Chlpls run through a distinct differentiation that justifies the use of an appropriate term as detailed by Sitte et al. (1980).

MATERIALS AND METHODS

Cultivation

Seedlings of *Brassica napus* L. cv Arabella were cultivated as described by Ginsburg and Matile (1993). After expansion and greening of cotyledons, 11 to 12 d after sowing, shoots were excised, the hypocotyls were placed in tap water, and senescence was induced upon incubation in permanent darkness for 4 d.

Isolation of Chlpls

The method of Mills and Joy (1980) was adapted as follows. Cotyledons (10 g fresh weight) were blended in a Sorvall Omni-mixer for 3 s at maximal speed in the presence of 40 mL of ice-cold homogenization medium (400 mM sorbitol, 55 mM Tricine-KOH buffer, pH 7.9, 2 mM Na-EDTA, 1 mM $MgCl_2$, 0.1% [w/v] BSA). The homogenate was filtered through a 25-μm nylon gauze and distributed equally to two 35-mL polycarbonate tubes, where it was underlayered with 4 mL of washing medium (363 mM sorbitol, 55 mM Tricine-KOH buffer, pH 7.9, 30% [v/v] Percoll) and a bottom layer of 2 mL of plain Percoll. After 90 s of centrifugation (3000*g*, swingout bucket), the intact Chlpls had layered at the interphase between the Percoll cushion and the washing layer. They were carefully removed, mixed with 10 mL of resuspension medium (363 mM sorbitol, 55 mM Tricine-KOH buffer, pH 7.9, 0.1% [w/v] BSA), and sedimented (5 min at 700*g*). The sediment was resuspended in the same medium to yield a density of Chlpls of about 2.5×10^9 mL^{-1} (counting in a hematocytometer). The preparations were stored on ice for no more than 3 h before use for assays. At least 80% of the organelles were intact as judged by the optical properties under phase contrast. The temperature was maintained at 0 to 4°C throughout the isolation procedure.

Separation of Stroma and Thylakoids

Suspensions of Chlpls were transferred to 1.5-mL Eppendorf tubes, frozen in liquid nitrogen, and subsequently thawed. Centrifugation in a Beckman microfuge (5 min at 13,000*g*) resulted in a transparent supernatant (stroma fraction) that was carefully removed and a green pellet that was resuspended in 20 mM Tris-Tricine buffer, pH 8.0. Stromal proteins were obtained by mixing stroma fraction with 4 volumes of precooled (−20°C) acetone. The sediment (2 min at 13,000*g*) was freed from the bulk of acetone by placing the tubes upside down in crushed ice for 5 min. It was resuspended in a volume of 20 mM Tris-Tricine buffer, pH 8.0, corresponding to the volume of the initial stroma fraction.

Preparation of Thylakoids from Homogenates

To simplify matters the preparation of washed, broken Chlpls described here was designated as thylakoids. Cotyledons (5 g fresh weight) were blended in a Sorval Omni-mixer two times for 5 s at full speed in the presence of 40 mL of the homogenization medium as used for the preparation of Chlpls, except for the addition of 10 mM Na-ascorbate im-

mediately before homogenization. The homogenate was passed through two layers of Miracloth and then centrifuged (3 min at 7,000*g*) in a Sorvall SS-34 rotor. The sediment was taken up in 1 mL of resuspension medium (400 mM sorbitol, 55 mM Tricine-KOH buffer, pH 7.9) and equally distributed among 10 1.5-mL Eppendorf tubes. The membranes were washed by adding 1 mL of resuspension medium, vortexing, and sedimenting (5 min at 13,000*g*). After removal of the supernatant medium, the tubes were frozen in liquid nitrogen and stored at −20°C for up to 1 week prior to use for assays.

Assay of the Cleavage Reaction

The system in organello comprised 30 μL of Chlpl suspension containing about 8×10^7 Chlpls and 1 μL of either ATP or Glc6P to yield final concentrations of 3 mM. The Eppendorf tubes were gently agitated to mix Chlpls and cofactors, warmed up to room temperature, and incubated in darkness for 1 h. The reaction was terminated by the addition of an equal volume of methanol and subsequent vortexing. In the case of the system in vitro, the final volume of the incubation mixtures was 35 μL. In terms of substrate and enzyme it contained either recombinations of thylakoidal and stromal fractions prepared from Chlpls or sediments of thylakoid resuspended in preparations of stromal protein. Alternatively, thylakoids isolated from homogenates were thawed and resuspended in 30 μL of 20 mM Tris-Tricine buffer, pH 8.0. These preparations were supplemented with NADPH (3 mM), Glc6P (3 mM), Glc6P-DH (10^{-2} units), and Fd (40 μg) to yield the final concentrations and dosages, respectively, given in parentheses. The assay mixtures were prepared on ice, vortexed, then warmed up to room temperature and incubated in darkness for 1 h. The reaction was stopped by the addition of an equal volume of methanol and subsequent vortexing. Prior to HPLC analysis the assay mixtures were centrifuged (5 s at 1300*g*) to remove proteins precipitated in the presence of 50% methanol.

HPLC of FCCs

The reverse-phase system that was employed has been detailed previously (Ginsburg and Matile, 1993). A major modification concerned the elution program with the solvents A (100 mM phosphate-K^+ buffer, pH 7, 50% methanol [v/v]) and B (100% methanol). It consisted of an initial gradient from A to B within 7 min, an isocratic run with B for 5 min, return to A within 3 min, and equilibration with A for 5 min. The volume injected was 20 μL and the flow rate was 1 mL min^{-1}. Fluorescence was recorded at 320 nm (excitation)/450 nm (emission). Bn-FCC-1 was eluted after 8 min, Bn-FCC-2 after 10.2 min. Amounts of FCCs are given in terms of integrated fluorescence intensities. So far, we have not been able to find a reference compound suitable for use as an internal standard. Moreover, the relationship between fluorescence yields and molar concentrations are unknown and will remain so until the purification of FCCs has been achieved on a preparative scale.

Radiolabeling of FCC-2 in Vitro

Cotyledons were radiolabeled with [4-^{14}C]5-aminolevulinic acid as described by Ginsburg and Matile (1993). Thylakoids were prepared from homogenates of cotyledons that had been induced to senesce in permanent darkness for 3 d and incubated in the presence of a stromal protein fraction and cofactors for 1 h. HPLC analysis was performed with sequential monitoring of fluorescence and radioactivity (Ginsburg and Matile, 1993).

Determinations

Chl was assessed in 80% acetone extracts using the coefficients provided by Lichtenthaler (1987). Protein-dye binding (Bradford, 1976) was employed for the determination of protein. Pheophorbide *a* was analyzed by HPLC as detailed by Langmeier et al. (1993).

Reproducibility

The lability of FCCs necessitated the performance of time-consuming HPLC analyses immediately after incubations. As a consequence, the parallel processing of more than six to eight assay mixtures was hardly possible. Therefore, the reproducibility of effects of various treatments was scrutinized by repetitions of experiments with different preparations of Chlpls at different times rather than by parallel incubations with the same preparation. Over a period of 3 years some of the basic experiments have been repeated dozens of times with excellent qualitative reproducibility.

Suppliers of Chemicals

NADPH, ATP, Glc6P-DH, and beef liver catalase were purchased from Sigma; Glc6P and Fd came from Fluka. Fd was also prepared from spinach leaves following the protocol recommended by Yocum (1982).

RESULTS

Production of Chl Catabolites in Organello

Intact senescent Chlpls contained a fluorescent compound, Bn-FCC-1, which had previously been identified as a derivative of Chl-porphyrin (Ginsburg and Matile, 1993). It was generated in organello when either Glc6P or ATP was supplied in the medium (Fig. 2). A second compound, Bn-FCC-2, was inconspicuous in the freshly prepared Gpls but increased markedly upon incubation in the presence of Glc6P or ATP. In the absence of cofactors, FCC-1 was partially lost, whereas FCC-2 remained practically constant. Chlpls from mature, green cotyledons were completely devoid of FCCs and failed to produce them in the presence of ATP or Glc6P (data not shown).

Production of Chl Catabolites in Vitro: Requirement for Reduced Fd

The lysis of Gpls abolished the capacity to generate FCCs; pools of catabolites did not increase when mixtures of thy-

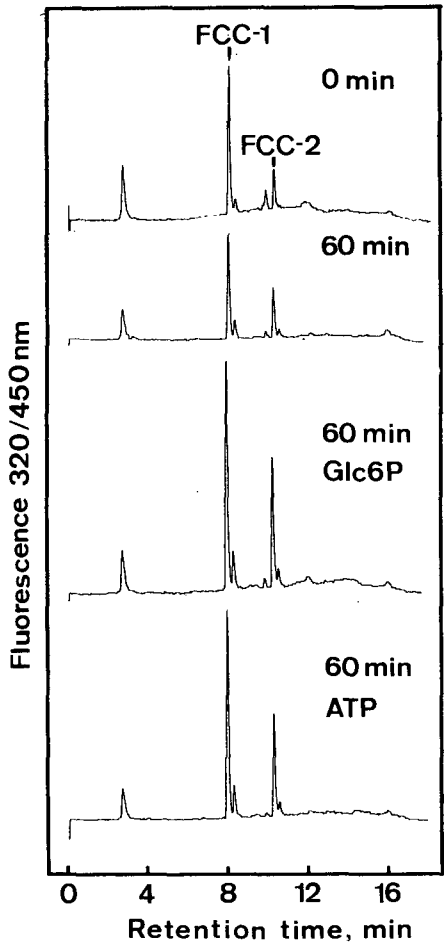

Figure 2. Generation of FCCs in intact rape Gpls is dependent on the supply of either Glc6P or ATP. The cotyledons employed for the isolation of Gpls had been weakly induced to senesce (exposure to continuous darkness for 2 d).

lakoids and soluble stromal components were incubated together with ATP and/or Glc6P (data not shown). We suspected that a decisive factor might be altered or diluted upon the disruption of Gpls and that this factor might be a reductant required for the operation of the redox cycle of the putative dioxygenase. In the end, we discovered that the presence of reduced Fd is a prerequisite for FCC production in the reconstituted system. This is documented by the data compiled in Table I. In contrast to the findings in organello (Fig. 2), the less polar of the two FCCs, Bn-FCC-2, was produced predominantly in the ruptured Gpls. The addition of NADPH was sufficient for the generation of some FCC-2. This effect was probably due to the presence in the assay mixture of endogenous Fd. Indeed, when thylakoids were incubated together with stromal proteins, the preparation devoid of Fd (50% acetone insolubles) was inactive unless exogenous Fd was added. Gpls contain NADP-Glc6P-DH activity (data not shown), which appears to be responsible for the marked positive effect of Glc6P supplied in conjunction with NADPH and Fd. Nevertheless, the addition of commercial Glc6P-DH caused a further increase of FCC production. In short, the optimal generation of FCC-2 in the reconstituted system came from the establishment of conditions aimed at keeping Fd in the reduced state. Moreover, reduced Fd could not be replaced by other cofactors known to be involved in reactions catalyzed by dioxygenases. Thus, ascorbate and 2-oxoglutarate (alone or in combination) did not support the generation of FCCs in vitro.

Identification of Bn-FCC2 as a Derivative of Chl-Porphyrin

The amount of FCC-2 in freshly prepared Gpls was too low for ^{14}C tracing as previously employed for the identification of FCC-1 as a Chl catabolite (Ginsburg and Matile, 1993). Therefore, we attempted to generate labeled FCC-2 in vitro by incubating thylakoids containing [^{14}C]Chl. The radiochromatograms of assay mixtures supplemented with Fd and cofactors demonstrated that by the end of incubation, a peak of ^{14}C coincided with the fluorescence signal of FCC-2

Table I. *Production of FCC-2 in vitro: requirement for reduced Fd*

Additions to Lysed Gpls[a]		ΔFCC-2 h^{-1}
None		0
NADPH		725[c]
NADPH + Fd		1330
Fd		0
NADPH + Glc6P + Fd		4645
NADPH + Glc6P + Glc6P-DH + Fd		5069
Additions to thylakoids[b]	**Fd**	**ΔFCC-2 h^{-1}**
50% acetone-insoluble stroma	−	0
	+	865
80% acetone-insoluble stroma	−	428
	+	3697

[a] Preparations of Gpls were lysed by freezing and thawing and incubated together with cofactors as described in "Materials and Methods." The cotyledons used for the isolation of Gpls had been induced to senesce in darkness for 4 d. [b] The reconstituted system consisted of thylakoids and stromal proteins from Gpls insoluble in 50 and 80% acetone, respectively. The precipitation of Fd requires a concentration of acetone of 75% (Shin, 1971). The cofactors NADPH, Glc6P, and Glc6P-DH were added throughout. [c] Integrated peak areas.

(Fig. 3). Incidentally, FCC-2 had spectral optical properties, including the conspicuous absorption maximum at 320 nm, which also typify several established catabolites of Chl-porphyrin (see Ginsburg and Matile, 1993).

Requirement for O_2

The time course of FCC production in an optimized system consisting of thylakoids, stroma fraction, Fd, NADPH, Glc6P, and Glc6P-DH is depicted in Figure 4. It appears that the reaction ran through a rapid initial phase and was nearly completed after about 20 min. This was unexpected considering an almost unlimited availability of Chl in the thylakoids and a generous supplementation of the assay mixture with reducing power. Yet, the kinetics can be explained by the tendency of Fd to reduce O_2 to H_2O_2 (Allen, 1975).

Keeping Fd in the reduced state must, therefore, inevitably cause depletion of O_2 and thereby interfere with the oxygenolytic cleavage reaction. This assumption was tested by including catalase in the assay mixture. Indeed, the continuous regeneration of O_2 resulted in a markedly enhanced production of FCC-2 (Table II). However, it did not bring about a prolonged formation of FCC-2, presumably because the increased availability of O_2 also provoked an accelerated consumption of Glc6P and NADPH. Hence, the reconstituted system is associated with an unsatisfactory, futile consumption of O_2. The effect of O_2 depletion was also demonstrated directly by the establishment of anoxic conditions in the assay mixture. Incubation in an atmosphere of argon in conjunction with enzymic removal of O_2 during incubation reduced the production of FCC-2 to about 2% of the production in the presence of O_2 (Table II).

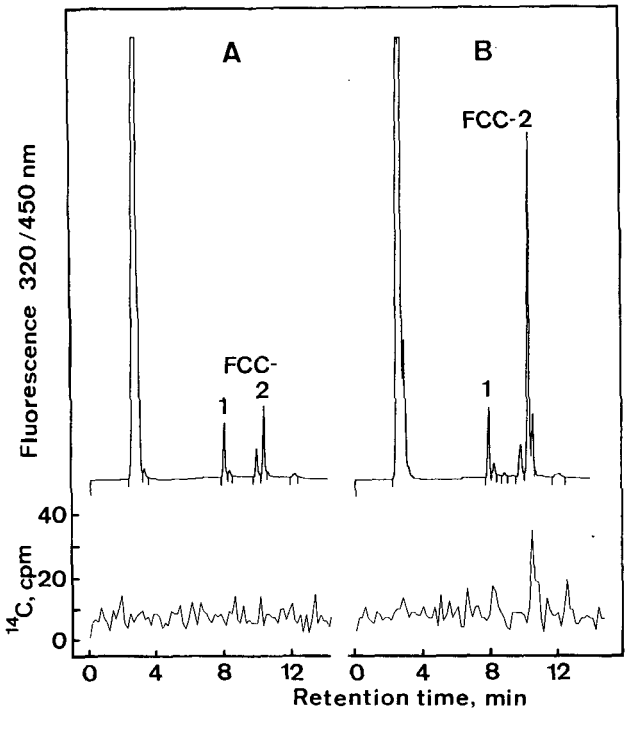

Figure 3. Identification of Bn-FCC-2 as a derivative of Chl-porphyrin. The reconstituted system comprised thylakoids radiolabeled as detailed by Ginsburg and Matile (1993) and isolated from cotyledons induced to senesce in darkness for 3 d, stroma fraction, Fd, NADPH, Glc6P, and Glc6P-DH. Extracts of the assay mixture in 50% methanol were chromatographed before incubation (A) and after 60 min (B). Fluorescence and radioactivity were monitored sequentially. The fluorescent compound eluted at the front is NADPH.

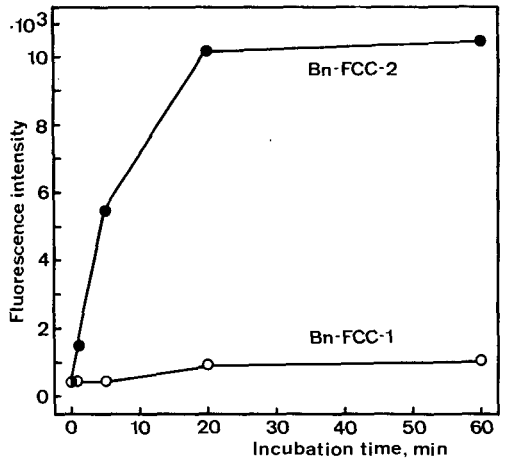

Figure 4. Time course of FCC production in a reconstituted system consisting of thylakoids, stroma proteins, Fd, and cofactors (NADPH, Glc6P, Glc6P-DH). Thylakoids were obtained from a homogenate of senescent cotyledons and the protein fraction was prepared by precipitation of stroma from Gpls with 80% acetone.

The time course shown in Figure 4 suggests that incubation periods should fall within the initial linear phase of the reaction (<10 min), when O_2 limitation has not yet occurred. Nevertheless, the assay mixtures were routinely incubated for 1 h because the final yield of FCCs was found to be proportional to the initial rate (see also Fig. 6B).

Requirement for Fe^{2+}

Cleavage of Chl-porphyrin in intact barley Gpls has been found to be strongly inhibited by chelators such as *o*-phenanthroline and 2,2′-dipyridyl (Schellenberg et al., 1990). This has been interpreted as indicating the involvement of Fe^{2+} in the oxygenolytic cleavage reaction. Corresponding inhibitions were also observed in the reconstituted system (Table

Table II. *The requirement of O_2 for the production of FCC-2 in vitro*

The complete system comprised thylakoids, stroma fraction, Fd, NADPH, Glc6P, and Glc6P-DH. Anoxic conditions were established by preincubation of the tubes in an atmosphere of argon prior to the addition of cofactors and by the inclusion of 2 mM Glc and 2 mg mL^{-1} Glc oxidase. The FCC-2 yield of control (9131 integrated fluorescence units) was taken as 100.

Treatments	ΔFCC-2 h^{-1}
Control	100
Anoxic conditions	2.2
Catalase (50 milliunits)	156

III). Of the various chelators tested, *o*-phenanthroline and 8-hydroxyquinoline were the most powerful inhibitors of FCC-2 production.

Thylakoids were also prepared from cotyledons that, during 3 d of dark-induced senescence, had been treated with 1 mM dipyridyl (see Langmeier et al., 1993). These membranes were inactive but slight reactivations were observed upon the pretreatment with, or incubation in the presence of, $FeSO_4$ (data not shown); since corresponding manipulations with Cu^{2+} or Zn^{2+} were entirely ineffective, we concluded that the oxygenolysis of Chl-porphyrin requires Fe^{2+} specifically.

The Contribution of Thylakoids

The system described so far consisted of either lysed Gpls or combinations of thylakoids and stroma fraction prepared from the same batch of Gpls. We wondered whether assays run with preparations from mature Chlpls, or combinations of thylakoids and stroma originating from Chlpls and Gpls, respectively, would reveal senescence-relevant contributions of one or the other of the components of the reconstituted system. A corresponding experiment is illustrated in Figure 5. The data demonstrate that both combinations containing senescent thylakoids were competent for the production of FCCs, whereas presenescent thylakoids failed to serve as a "substrate," whether they were incubated in the presence of stroma from Chlpls or from Gpls. The contributions of both stroma and senescent thylakoids were heat labile (Fig. 5), suggesting that enzymes located in both compartments contribute equally to the overall reaction. We also concluded that the pace-setting part of the breakdown pathway resides in the thylakoids.

Senescent thylakoids contained small pools of pheophorbide *a*, which tended to increase during incubations. The accumulation of pheophorbide *a* was markedly smaller when FCC-2 was allowed to be produced in a reconstituted system compared to incubations in the absence of Fd and cofactors (Table IV). We tentatively concluded that pheophorbide is the substrate of the stromal dioxygenase. However, only thylakoids were suitable substrates; free pheophorbides did not yield FCCs if they were incubated together with stroma and the usual blend of cofactors.

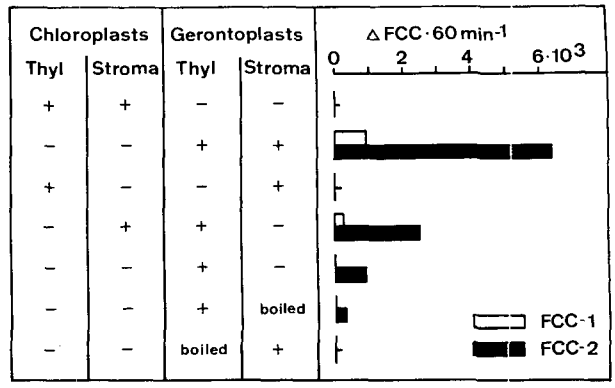

Figure 5. Production of FCCs in reconstituted systems comprising the four combinations of thylakoids (Thyl) and stroma fractions from mature Chlpls and Gpls. Cofactors (Fd, NADPH, Glc6P, Glc6P-DH) were added throughout. Heat treatment was 30 s in a boiling-water bath.

FCC-2 Production in Thylakoids

Senescent thylakoids had a small capacity to produce FCC-2 in the absence of stroma fraction (Fig. 5), suggesting that the stromal part of the reconstituted system is not easily released upon the lysis of Gpls. Repeated washing of the membranes with buffer removed most residual activity, but the stripped thylakoids retained the competence to produce FCCs if they were incubated together with stroma (data not shown). Gpls appear to contain an excess of the stromal factor, as can be inferred from experiments in which a constant amount of thylakoids was challenged with decreasing doses of stroma (Fig. 6A). A marked reduction of FCC production was observed only upon additions of comparatively small amounts of stroma.

Attempts to simplify the protocol for the preparation of competent thylakoids have led to the observation that thy-

Table III. *Effects of various chelators on the production of FCC-2*

The assay mixtures contained thylakoids prepared from homogenized senescent cotyledons as described in "Materials and Methods" (see also Fig. 7), Fd, the standard blend of cofactors, and 1% DMSO, the solvent of apolar chelators. Na-EDTA and Na-salicylate were dissolved in water and the corresponding controls contained no DMSO. Fluorescence units of FCC-2 integrated in controls were taken as 100. Numbers of independent determinations are given in parentheses.

Chelator	Concentration (mM)	ΔFCC-2 h^{-1}
None		100
o-Phenanthroline	1	2
	2.5	0
8-Hydroxyquinoline	1	0
2,2′-Dipyridyl	2.5	70 ± 5 (3)
	5	29 ± 6 (3)
Diethyldithiocarbamate	5	47 ± 6 (3)
EDTA	2.5	80 ± 8 (5)
Salicylate	2.5	37 ± 14 (4)

Table IV. *Inverse relationship between the production of FCC-2 and the accumulation of pheophorbide a*

The experiment was performed with "loaded" thylakoids (see Fig. 7); analogous incubations of reconstituted systems (thylakoids + stroma fraction) demonstrated the same inverse relationship. Cofactors were NADPH, Glc6P, Glc6P-DH, and Fd. Thylakoids (equivalent to 10^8 Gpls per assay mixture) were isolated from cotyledons induced to senesce in darkness for 4 d.

Additions	ΔFCC-2 h^{-1} (integrated peak area)	ΔPheophorbide h^{-1} (nmol)
None	0	1.94
Cofactors	5559	0.72
Cofactors + 2.5 mM o-phenanthroline	0	2.73

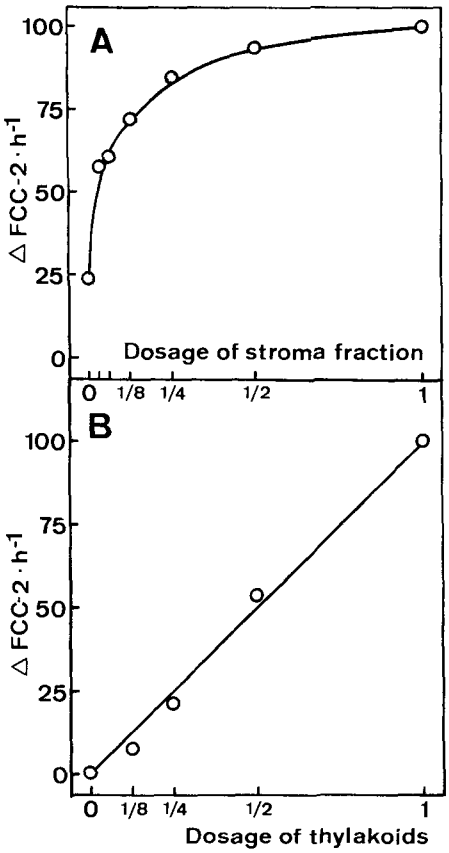

Figure 6. A, Production of FCC-2 in isolated thylakoids as a function of the dosage of stroma fraction. Gpls from cotyledons (3 d, permanent darkness) were lysed using a freeze-thaw cycle and divided into thylakoids and stroma fraction. Equal amounts of thylakoids were present in all assay mixtures. B, Production of FCC-2 as dependent on the dosage of thylakoids. The membranes "loaded" with stromal factor(s) were prepared from homogenates of senescent cotyledons. The assay mixtures contained the standard blend of Fd and cofactors. FCC-2 production is given as relative equivalents of integrated fluorescence units.

lakoids "loaded" with FCC-producing activity can be obtained directly from homogenates if a buffered sorbitol medium is employed. Na-ascorbate was an additional and essential ingredient of the homogenization medium. Such "loaded" thylakoids appeared to be saturated with the stromal part of the system: supplementation with additional stroma caused no further increase of FCC production (data not shown). As shown in Figure 6B, the production of FCC-2 was proportional to the amount of loaded thylakoids in the assay mixture. Thylakoids prepared from mature, green cotyledons were inactive, although the Chpls contain the stromal part. As shown in Figure 7, the activity appeared and rapidly increased in the loaded thylakoids as senescence was induced upon the exposure of excised shoots to permanent darkness. It peaked when about half of the Chl had been broken down in the cotyledons and thereafter declined toward the end of the senescence process. Treatments of the cotyledons with an inhibitor of cytoplasmic protein synthesis, D-MDMP, significantly reduced the FCC-producing activity in the thylakoids (Table V). MDMP was chosen because the availability of the ineffective L-isomer allows the exclusion of nonspecific side effects (Thomas, 1976). Since the system of thylakoids and stromal factor(s) associated with them is quite complex, the component(s) depending on cytoplasmic protein synthesis cannot, for the time being, be identified or localized.

Requirement for ATP?

In the intact Gpls the supply of exogenous Glc6P provoked the breakdown of Chl to FCCs (Fig. 2; Matile et al., 1992). This effect can be explained with the requirement for reduced Fd, as demonstrated in the reconstituted system of thylakoids and stroma (Table I). However, in the intact Gpls, Glc6P can be replaced by ATP (Fig. 2; Schellenberg et al., 1990); it is disappointing to state that in the reconstituted system, ATP had no significant effect, whether given alone or in conjunction with Fd and cofactors (data not shown). It can only be

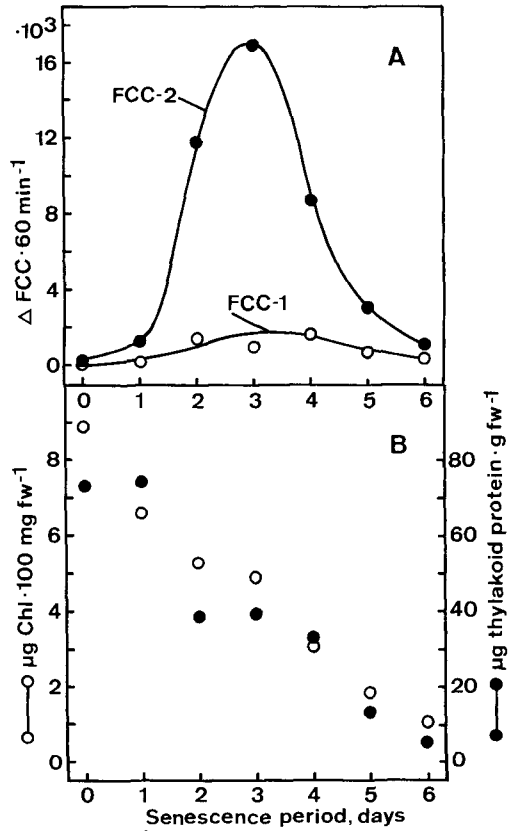

Figure 7. The production of FCCs in vitro by "loaded" thylakoids isolated from homogenates of cotyledons at various stages of senescence. A, FCC production in the presence of Fd and the standard blend of cofactors. B, Contents of total Chl in the cotyledons and amount of protein recovered in the preparations of thylakoids. At each stage of dark-induced senescence, cotyledons (5 g fresh weight) were homogenized and loaded thylakoids were prepared as described in "Materials and Methods." The data on FCC production (integrated fluorescence intensities) refer to the thylakoid equivalent of 1 g of cotyledon tissue.

Table V. *Effect of D-MDMP, an inhibitor of cytoplasmic protein synthesis on FCC-2 production, in "loaded" thylakoids*

Shoots of rape seedlings were excised on d 10 after sowing and the hypocotyls were placed in 10^{-5} M solutions of MDMP made up in tap water and incubated in darkness for 3 d prior to the preparation of thylakoids from cotyledons. The assay mixtures contained the standard blend of cofactors. Data are presented as integrated fluorescence units.

Effector	ΔFCC-2 60 min^{-1}
None	1640
D-MDMP	585
L-MDMP	2106

speculated that in the intact organelles, ATP is responsible for the activation of an endogenous substrate that eventually is employed for the reduction of Fd.

DISCUSSION

One of the difficulties of tackling the mechanism of Chl breakdown is the lack of knowledge about the structure of the primary products(s) of ring opening. We assume that one of the FCCs produced in rape Gpls is a direct derivative of pheophorbide *a* and, hence, may be addressed as the counterpart of biliverdin, the cleavage product of heme. The FCCs occur in such minute amounts and, in addition, are so labile, that it would be hopeless to strive for purification on a preparative scale. Therefore, the mechanism of cleavage can only be inferred indirectly from the structure of Chl catabolites, which, in senescent leaves, accumulate in sufficiently large quantities.

The NCCs are likely to represent derivatives of the primary FCCs. Available structures of NCCs suggest that the catabolic system of plants is different from mammalian heme oxygenase. Although in heme as well as in Chl cleavage of the macrocycle takes place in the α position, the methene carbon is released in the case of heme (Tenhunen et al., 1969), whereas in the NCCs it is preserved as a formyl residue attached to pyrrole B (Kräutler et al., 1991, 1992; Mühlecker et al., 1993). Heme oxygenase is a Cyt P450 monooxygenase that consumes 3 mol of molecular O_2 for the oxidation of the α-methene carbon to CO and for two hydroxylations that take place during ring opening; the release of Fe^{3+} is a consequence of the cleavage reaction. In contrast, the cleavage of Chl-porphyrin appears to be preceded by the enzymic removal of Mg^{2+} (Langmeier et al., 1993), and oxygenolysis is likely to require pheophorbide as substrate. Incidentally, a participation of a Cyt P450-type of oxygenase can be ruled out, since CO did not inhibit the production of FCCs in intact rape Gpls (S. Ginsburg, unpublished results). Formally, the cleavage of Chl-porphyrin can be conceived as the addition of molecular O_2 and, hence, the enzyme concerned can be apostrophized as a dioxygenase.

In plants, most oxygenating enzymes described so far are monooxygenases or dioxygenases that require two substrates, one of them being, for example, 2-oxoglutarate (for reviews, see Butt and Lamb, 1981; Prescott, 1993). Two examples of truly oxygenolytic cleavage concern aromatic compounds: a dioxygenase demonstrated to occur in many species that catalyzes the cleavage of homogentisic acid (Durand and Zenk, 1974a, 1974b) and 2,3 dihydroxybenzoic acid dioxygenase in leaves of *Tecoma stans* (Sharma and Vaidyanathan, 1975). The latter is a Cu^{2+}-containing protein; in our context it is interesting because it appears to be located in the Chlpls. The former enzyme is Fe^{2+} dependent as demonstrated by the inhibitory effect of 2,2′-dipyridyl (Durand and Zenk, 1974b).

Evidence presented here favors the existence in rape Gpls of a (pheophorbide)-dioxygenase, which also depends on Fe^{2+}. Rather surprisingly, it appears to require reduced Fd as reductant, sharing this property with choline monooxygenase, a stromal enzyme of spinach Chlpls involved in the biosynthesis of betaine (Brouquisse et al., 1989). It may be argued that the requirement of Fd, although an interesting feature of our reconstituted system, is irrelevant for Chl breakdown in vivo because Fd is likely to be lost in senescent leaves as rapidly as other soluble Chlpl proteins. Yet, preliminary assessments by immunoblotting have suggested that in barley leaves Fd is largely retained throughout the main period of Chl degradation (H. Thomas, unpublished results). This may be due to senescence-induced synthesis of Fd making up for losses through general degradation of stromal proteins. Indeed, in maize one of several senescence specifically expressed genes has been identified with a gene encoding Fd (C. Smart, unpublished results). This finding suggests quite strongly that Fd is required for metabolic processes in senescent leaves, and we propose that oxygenolytic cleavage of Chl-porphyrin is one of them.

It is interesting to note that a heme oxygenase that in the red alga *Cyanidium caldarium* catalyzes the first step of phycobilin formation also requires reduced Fd (Rhie and Beale, 1992). Although the mechanisms of ring cleavage are probably different in the formation of biliverdin from protoheme and of FCC from pheophorbide, the common requirement of reduced Fd is intriguing.

Present knowledge about Chl breakdown suggests the sequential action of at least three enzymes: (a) chlorophyllase hydrolyzing Chl to Chlide; (b) Mg-dechelatase converting Chlide to pheophorbide; and (c) dioxygenase responsible for oxygenolytic formation of an FCC. A detailed analysis of this pathway, especially of the last step, is hampered by the fact that senescent thylakoids are indispensable as a complex substrate. In senescent rape cotyledons, pheophorbide *a* is accumulated if cleavage is inhibited in the presence of dipyridyl (Langmeier et al., 1993), yet it is not released from the thylakoids but remains associated with the apoproteins of light-harvesting complex II (M. Langmeier, M. Schellenberg, P. Matile, unpublished results). It appears that the oxygenating enzyme recognizes the complexed substrate within the membrane rather than free pheophorbide. Although we assume that dioxygenase is the stromal factor of our reconstituted system, this remains to be demonstrated, and we admit that even the participation in the overall reaction from pheophorbide to FCC of additional protein(s), such as a protease, cannot be excluded. In any case, a more detailed analysis of the system will be indispensable for the purification of relevant catalysts such as Mg-dechelatase and dioxygenase.

Still another problem concerns the interpretation of FCCs

with regard to their position in the pathway. In the case of barley Gpls, three FCCs have been detected upon studies of Chl breakdown in organello (Schellenberg et al., 1990; Matile et al., 1992) and in a reconstituted system (Schellenberg et al., 1993). In rape there are only two FCCs, but it is difficult to decide whether only one of them or both should be addressed as direct product(s) of cleavage. Because there are two substrates, Chl *a* and Chl *b*, the more polar FCC could be considered a derivative of the more polar substrate, Chl *b*, but there are other possibilities, such as two different cleavage reactions or one of the FCCs being derived from the other. We favor the view that Bn-FCC-2 is the primary derivative of pheophorbide *a* because, as judged by polarities on reverse-phase HPLC, very similar if not identical FCCs are produced in Gpls of barley (Hv-FCC-4, Schellenberg et al., 1993) as well as of spinach (F. Vicentini, unpublished result). These FCCs are the most apolar of the series, and the higher polarities of other FCCs may be due to hydroxylations in side chains and other species-specific modifications as suggested by the structures of Hv-NCC[RP14] (Kräutler et al., 1991, 1992) and Bn-NCC-1 (Mühlecker et al., 1993). It should be pointed out that except for the apparently common FCCs, all other catabolites so far identified in several species are different. We tentatively conclude that these findings point to a common mechanism of cleavage yielding a common FCC that is modified in a species-specific fashion, eventually resulting in a species-specific set of secondary catabolites. Regarding the duality of potential substrates, we originally expected to find couples of catabolites, one originating from Chl *a*, the other from Chl *b*. We have failed so far to confirm this expectation. Both catabolites that have been structurally analyzed are derivatives of Chl *a* and, thus, the fate of Chl *b* will probably require an investigation directed to this specific objective.

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