Breakdown of thylakoid pigments by soluble proteins of developing chloroplasts

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In the presence of Triton X-100 (TX-100) or imazalil, plastidic pigments were degraded by a soluble enzyme extracted from developing chloroplasts. This bleaching was not photochemical and required oxygen; it was not inhibited by superoxide dismutase or catalase, but was strongly inhibited by benzoquinone, quinol, phenazine methosulphate and, more weakly, by other reagents. Synthetic intermediates of chlorophyll biosynthesis, e.g. Mg(II)-protoporphyrin IX monomethyl ester, was also degraded. This reaction was compared with the bleaching catalysed by soybean (*Glycine max*) lipoxygenase. The plastidic system required TX-100 and was inhibited by unsaturated fatty

acids, whereas lipoxygenase required a polyunsaturated fatty acid and was inhibited by TX-100. The bleaching capability of the stromal extract decreased with age if the seedlings were placed in the greenhouse to allow further development of the chloroplasts. A direct relationship was observed between the promotion of pigment bleaching by TX-100 and the inhibition of the *in vitro* synthesis of divinylprotochlorophyllide. This bleaching reaction is discussed on the basis of interference by TX-100 with the normal O₂-requiring anabolic processes of developing chloroplasts.

INTRODUCTION

The main research focus in our laboratory has been the study of chlorophyll synthesis in developing chloroplasts, with special attention to the oxidative cyclization of Mg(II)-protoporphyrin IX monomethyl ester (MgProtoMe) to yield divinylprotochlorophyllide (DVPchlde) (Walker et al., 1991, and references therein). However, it has been observed that greening tissues and their subcellular isolates can also bring about the degradation of chlorophyll (Hendry and Stobart, 1986; Stobart and Hendry, 1984) and of chlorophyll precursors (Gassman and Ramanujam, 1986).

In our study of the MgProtoMe (oxidative) cyclase, we noticed that the sum of substrate (MgProtoMe) and product (DVPchlde) was not always constant. This variability led us to investigate pigment breakdown under the conditions used in our cyclase assay (Walker et al., 1991; Vijayan et al., 1992). The starting point for this investigation was the observation that imazalil, an inhibitor of lanosterol 14 α -demethylase (Baldwin, 1983, 1990), and therefore a putative inhibitor of cytochrome P-450, inhibited also the MgProtoMe (oxidative) cyclase system (Table 1 of Whyte and Castelfranco, 1993). We soon discovered, however, that imazalil also caused the destruction of endogenous thylakoid pigments. This property was not confined to imazalil; other semipolar organic molecules appeared to do the same, and, as we shall see, Triton X-100 (TX-100) was a particularly strong activator of pigment degradation.

In the present study, we describe an '*in vitro*' subplastidic membrane pigment breakdown system activated by TX-100. Chlorophyll breakdown has been reviewed recently by Brown et al. (1991) and Hendry et al. (1987). The original literature is

extensive and confusing. Evidence has been presented for four types of reactions that may contribute to chlorophyll breakdown *in vivo*, namely photochemical processes, H₂O₂-dependent reactions collectively denoted by the term 'chlorophyll peroxidase', chlorophyll oxidation linked to the peroxidation of unsaturated fatty acids by the enzyme lipoxygenase and other O₂-dependent chlorophyll breakdown reactions denoted by the term 'chlorophyll oxidase'. Our own system appears to be different from all these types.

In the present paper we are particularly interested in relating the activity of our own subplastidic pigment breakdown system to the developmental stage of the plastids from which it is derived and to the ability of those plastids to carry out the oxidative cyclization of MgProtoMe, a step in the biosynthesis of chlorophyll.

MATERIALS AND METHODS

Materials

Chemicals

Catalase, CHAPS, cetyltrimethylammonium bromide (CTAB), cysteine hydrochloride, diethyldithiocarbamic acid sodium salt (DIECA), 4,5 dihydroxy-1,3-benzenedisulphonic acid, n-dodecyl β -D-maltoside, EDTA, glucose 6-phosphate disodium salt (Glc-6-P), glucose-6-phosphate dehydrogenase (Glc-6-PDH; type XII from *Torula* yeast, EC 1.1.1.49), Hepes, lipoxygenase (type 1-B from soybean; EC 1.13.11.12), linoleic acid (LLA), linolenic acid (LNA), n-octyl glucoside, oleic acid, phenazine methosulphate (PMS), Tes, Methyl Viologen, MgCl₂, NADP⁺, Percoll, S-adenosyl-L-methionine chloride salt (SAM), SDS, sodium

Abbreviations used: CTAB, cetyltrimethylammonium bromide; DIECA, diethyldithiocarbamic acid; DMSO, dimethyl sulphoxide; DV, divinyl; Glc-6-P, glucose 6-phosphate; Glc-6-PDH, glucose-6-phosphate dehydrogenase; imazalil, $\pm$ 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1H-imidazole; LLA, linoleic acid; LNA, linolenic acid; MgProtoMe, Mg(II)-protoporphyrin IX monomethyl ester; MgKPM, Mg(II)-7-(2-carboxyethyl)-4-ethyl-1,3,5,8-tetramethyl-6-[(1-oxo-2-methoxycarbonyl)ethyl]-2-vinylporphyrin, the 6-methyl β -oxopropionate analogue of MgProtoMe (2-vinyl, 4-ethyl-substituted); p.a.r., photosynthetically active radiation; Pchlde, protochlorophyllide; PMS, phenazine methosulphate; SAM, S-adenosyl-L-methionine; SOD, superoxide dismutase; TX-100, Triton X-100.

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cholate, sorbitol, superoxide dismutase (SOD) and TX-100 were purchased from Sigma.

Acetone, $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade), dimethyl sulphoxide (DMSO), hexanes and diethyl ether (for anaesthesia) were obtained from Fisher.

BSA was obtained from Calbiochem. KCN was from Mallinckrodt. Quinol and Methylene Blue were from Matheson, Coleman and Bell. Tween 80 was from ICI Americas. *p*-Benzoquinone was from Eastman Kodak. Resorcinol was from Merck. Imazalil was kindly given by Janssen Pharmaceuticals. TX-100 (low carbonyl and peroxide) was purchased from Pierce Chemical Co. TX-114 was obtained from Fluka. Desferal mesylate was a gift from CIBA Pharmaceutical Co.

Mg-porphyrin substrates

Synthetic MgProtoMe, used as the routine substrate for the reconstituted cyclase, was prepared by the method of Shiau et al. (1991). Mg(II)-7-(2-carboxyethyl)-4-ethyl-1,3,5,8-tetramethyl-6-[(1-oxo-2-methoxycarbonyl)ethyl]-2-vinylporphyrin (MgKPMe), the 6-methyl β -oxopropionate analogue of MgProtoMe, was prepared as described by Cox et al. (1974). Stock solutions were prepared at 333 μM concentration in DMSO.

Plant material

Cucumber (*Cucumis sativus* L. cv. Beit Alpha MR) seeds were obtained from Sunseeds Co., Hollister, CA, U.S.A. Seeds were germinated in the dark at 28–30 °C for 6 days. If chloroplasts were required, seedlings were exposed to white light [60–80 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetically active radiation (p.a.r.) at 28–30 °C] for 20 h before plastid isolation.

Methods

Buffers

Plastids were isolated in hypertonic buffer A (10 mM Hepes/20 mM Tes/1 mM MgCl_2 /2 mM sodium EDTA/0.5 M sorbitol, pH 7.7). Lysis was achieved using hypotonic buffer B (10 mM Hepes/20 mM Tes/1 mM MgCl_2 /2 mM sodium EDTA, pH 7.7).

Plastid isolation and preparation of subplastidic components

Intact chloroplasts, or etioplasts, were isolated as described by Fuesler et al. (1984). Cucumber chloroplasts were resolved into pellet and supernatant components by centrifugation at 12000 *g* after lysis in buffer B for 10 min at 0–4 °C. The 12000 *g* supernatant was cloudy, but the cloudiness did not interfere with our normal spectrophotometric assay (see below). A perfectly clear fluid was obtained by centrifuging the 12000 *g* supernatant for 30 min at 100000 *g*.

Pigment extraction into organic solvents

Thylakoid membranes from the usual 20 h-greened cucumber plastids were extracted with 80% (v/v) acetone containing 10 mM NH_4OH . The acetone extract was partitioned with hexane, and the coloured upper phase was dried under reduced

pressure in the dark with a rotary evaporator. The residue was redissolved in water containing TX-100. A volume of the pigment solution in the aqueous detergent was added to the assay mixture such that the final TX-100 concentration was 1 mM.

Incubation conditions

All incubations were carried out at 30 °C in the dark.

1. MgProtoMe (oxidative) cyclase. Cyclase incubations were carried out for 60 min in 1 ml of buffer as previously described (Walker et al., 1991). Hypertonic buffer A was used in all incubations with intact etioplasts and developing chloroplasts. Synthetic MgProtoMe was used as the substrate. The product, DVPchl_{ide}, was extracted into diethyl ether (Castelfranco et al., 1979) and assayed fluorimetrically as described by Chereskin et al. (1982). NADPH was added as a regenerating system [6.5 mM glucose 6-phosphate (Glc-6-P)/0.8 mM NADP^+ /Glc-6-PDH (1.1 units).]

2. Pigment-bleaching assay. Routine incubations for the bleaching assay were carried out in a 1 cm-pathlength spectrophotometer cuvette containing 3.0 ml of buffer A for intact plastids or 3.0 ml buffer B for subplastidic constituents. Routine incubation mixtures included intact plastids (3.0–8.0 mg of protein) or supernatant (2.5–5.0 mg of protein), plus pellet (1.0–2.5 mg of protein) fractions and 1 mM TX-100. The cuvette was stirred automatically in the dark. Departures from this general protocol are indicated in the Table and Figure legends. Absorption spectra were recorded automatically, usually every 10 min, using an SLM-Aminco DW2000 spectrophotometer in the split-beam mode (Vijayan et al., 1992). Data were converted into ASCII format and plotted using a Sigmaplot 4 graphics program on an IBM computer. Quantification of pigment changes for tabulation purposes was done at 672 nm (chlorophyll *a* red peak) or 632 (Pchl_{ide} red peak). The reference wavelength was 720 nm.

Exogenous fatty acids and other hydrophobic components were added in ethanol solution, which was kept below 2% in the final incubation. At this concentration, ethanol had no effect on the course of the bleaching. Equivalent amounts of ethanol were added to the controls.

3. Anaerobic incubations. Anaerobic incubations for the pigment-bleaching assay were carried out in a 25 ml Erlenmeyer flask wrapped in aluminium foil while Ar was flushed continuously through the system (Huang and Castelfranco, 1988). At the end of 30 min, the contents were transferred to the spectrophotometric cuvette, scanned, and the spectrum compared with the initial one recorded before the anaerobic incubation. A control sample was treated in the same manner, but flushed with air instead of Ar. The extent of anoxia achieved was not monitored. However, the synthesis of 5-aminolaevulinic acid by intact developing chloroplasts in the light was inhibited by approx. 90% (Huang and Castelfranco, 1988), showing that even the photosynthetically produced O_2 was flushed out. This method avoids the addition of foreign substances like glucose and glucose oxidase into the incubation medium.

Heat-inactivation of supernatant

Supernatant was prepared by osmotic lysis as described above. Portions (1 ml) were boiled for 5 min in Corex tubes in a heated water bath. The contents were allowed to cool, and the coagulated proteins were resuspended with a transfer pipette prior to use in the bleaching assay.

Protein determination

Protein was determined by the method of Bradford (1976), with BSA as standard.

RESULTS

Figures 1 and 2 show the effect of TX-100 on isolated etioplasts and developing chloroplasts respectively. Spectral decreases are apparent in both red and blue regions of the spectrum. Imazalil

Figure 1 TX-100-induced pigment bleaching in intact etioplasts

The cuvette contained 7.8 mg of etioplast protein in buffer A; 1 mM (0.06% v/v) TX-100 was added. Cuvettes were stirred in the dark at 30 °C, and absorption spectra were recorded automatically every 10 min.

Figure 2 TX-100-induced pigment bleaching in intact chloroplasts

The cuvette contained 3.6 mg of chloroplast protein in buffer A; 1 mM TX-100 was added. Spectra were recorded as in Figure 1.

Figure 3 Effect of (a) TX-100 and (b) imazalil on cyclase and pigment-bleaching reactions in intact etioplasts

Cyclase assays (○): 1 ml of reaction mixture contained 10 μM MgProtoMe, 1 mM SAM and an NADPH-regenerating system (see the Materials and methods section). Etioplast protein was 1.9 mg in (a) and 2.1 mg in (b). Different concentrations of TX-100 or imazalil were added and Pchlide was estimated after 1 h as described in the Material and methods section. Pigment bleaching assays (●): 3 ml of reaction mixture contained 5.7 mg of etioplast protein in (a) and 6.3 mg in (b). Different concentrations of TX-100 or imazalil were added, and absorption spectra were recorded every 10 min. The reference wavelength was 720 nm. At each concentration the absorbance decrease at 632 nm (over 30 min) was calculated. This value was divided by the absorbance decrease at 632 nm (over 30 min) for 1 mM Triton or imazalil (i.e. arbitrary maximal bleaching). The resulting value was multiplied by 100.

gave qualitatively similar spectral changes. Experiments in which both pigment bleaching and cyclase activities were measured simultaneously indicated a strong correlation between endogenous pigment destruction and cyclase inhibition for both imazalil and TX-100 (Figures 3 and 4).

Properties of TX-100-stimulated pigment breakdown

Properties of the TX-100-dependent pigment breakdown system were investigated further using the reconstituted chloroplast system. Osmotically lysed chloroplasts yield pellet and supernatant fractions, both of which are required for cyclase activity (Wong & Castelfranco, 1984; Walker et al., 1991). Pigment bleaching was also very active under these conditions (results not shown), but heat-inactivation experiments revealed that only the supernatant contained the enzymic activity (Table 1). In this and other Tables, the numerical values were calculated from 30 min and 0 min spectral data. However, in all cases where stimulation or inhibition of the bleaching rates is recorded, we observed that these effects were already noticeable in the 10 min spectra and remained roughly proportional throughout the experimental run.

Anoxic conditions inhibited the pigment breakdown (Figure 5). Exogenous NADPH and Mg^{2+} were not required for the observed pigment degradation (cf. Whyte et al., 1992). The reaction proceeded in complete darkness, but was totally inhibited at 0–4 °C (results not shown). Supernatant-associated activity could be precipitated with 85% satd. $(NH_4)_2SO_4$ or aq. 85% (v/v) acetone. Optimal conditions for these precipitations were not determined.

The pigment-bleaching system showed an absolute requirement

Figure 4 Effect of (a) TX-100 and (b) imazalil on cyclase and pigment-bleaching reactions in intact chloroplasts

Cyclase (○) and pigment bleaching (●) assays were carried out as for Figure 3. Chloroplast protein was 2.9 mg/ml in (a) and 3.0 mg/ml in (b) for both assays. The reference wavelength was 720 nm. At each concentration, the absorbance decrease at 672 nm (over 30 min) was calculated. This value was divided by the absorbance decrease at 672 nm (over 30 min) for 1 mM Triton or imazalil (i.e., arbitrary maximal bleaching). The resulting value was multiplied by 100.

Table 1 Heat-instability of the supernatant component and requirement for TX-100

Unboiled supernatant (2.9 mg of protein) or boiled supernatant (3.2 mg of protein) were incubated separately with the thylakoid pellet (1.2 mg of protein) with or without 1 mM TX-100. The decrease in absorbance at 672 nm was calculated from 0 min and 30 min spectra normalized at 720 nm.

Sample	$10^3 \times$ Decrease in A_{672}/min
Pellet + supernatant (no TX-100)	0
Pellet + supernatant + 1 mM TX-100	18.0 ± 0.2
Pellet + boiled supernatant + 1 mM TX-100	0.2 ± 0.1

for TX-100 (Table 1; Figure 6). A range of detergents were tested for their ability to induce a similar pigment-bleaching reaction. At a concentration of 1 mM, SDS, dodecyl maltoside and TX-114 were mildly effective (Figure 6); other detergents, namely CHAPS, CTAB, Tween 80, sodium cholate and n-octyl glucoside, were totally ineffective (results not shown).

TX-100-induced loss of pigments, as revealed by direct absorption spectroscopy of thylakoid membranes, was likewise apparent after extraction of the $t = 0$ min and $t = 30$ min samples into aq. 80% acetone. The time-dependent decrease in absorbance of suspensions therefore reflects genuine pigment breakdown and not merely changes in the physical properties of the microenvironment. The supernatant component of the reconstituted system caused degradation, not only of the pigments associated with plastidic membranes, but also of the same

Figure 5 Absorbance difference spectra showing the effect of anoxia on the pigment-bleaching reaction for the reconstituted chloroplast system

Subplastidic fractions, 1.3 mg of pellet and 2.4 mg of supernatant protein, were incubated with 1 mM TX-100 for 30 min at 30 °C. Difference spectra (30 min minus zero time) were obtained for an incubation in argon and an incubation in air.

Figure 6 Effect of different detergents on pigment-bleaching reaction

A 1 mg portion of chloroplast pellet protein and 3.1 mg of supernatant (snt) protein were incubated at 30 °C for 30 min in the presence of various detergents (1 mM). The control is the complete incubation mixture without the detergent. The next entry is pellet + 1 mM TX-100 (no supernatant) under the same conditions. The change in absorbance at 672 nm after 30 min was calculated from the resulting absorption spectra.

Table 2 Degradation of solvent-extracted thylakoid pigments by chloroplast supernatant

The pigments were extracted with organic solvents as described in the Materials and methods section and resuspended in water containing TX-100. A volume of this aqueous pigment solution was added to the cuvette to give a final TX-100 concentration of 1 mM. Supernatant (3.3 mg of protein) was added to one sample. The decrease in absorbance at 672 nm was calculated from the resulting $t = 30$ min and $t = 0$ min spectra, taking 720 nm as the reference wavelength.

Sample	$10^3 \times$ Decrease in A_{672}/min
Pigments + TX-100 (no supernatant)	0.07 ± 0.03
Pigments + TX-100 + supernatant	14.65 ± 0.75

pigments that had been extracted into organic solvents prior to addition to the incubation mixture (Table 2).

Moreover, this system could degrade the exogenous pigments

Figure 7 Degradation of exogenous MgProtoMe by chloroplast supernatant component

The cuvette contained 3.3 mg of chloroplast supernatant protein, 10 μ M MgProtoMe (in 90 μ l of DMSO) to a final volume of 3 ml with buffer B; 1 mM TX-100 was added, and absorption spectra were recorded every 10 min.

Figure 8 Effect of plant age on pigment-bleaching activity of chloroplast supernatant fractions

Chloroplasts were isolated from dark-germinated cucumber seedlings, light-exposed for 20 h, which had been placed in the greenhouse for 0, 1, 3 and 7 days. Supernatant fractions were isolated by lysis (as described in the Materials and methods section) and stored at -80°C . Supernatants were assayed simultaneously using the same 20 h-greened pellet (1.7 mg of protein) as the source of pigments. Absorption spectra were recorded every 10 min at 30°C , and the decrease in absorbance at 672 nm was calculated. The 'pellet alone (control)' sample (curve close to the abscissa) contained pellet and detergent, but no supernatant.

used routinely as substrates for the cyclase reaction. Figure 7 shows degradation of MgProtoMe in the presence of the supernatant. The 6-methyl β -oxopropionate derivative, MgKPM, was also broken down under these conditions (results not shown). TX-100 was likewise absolutely required for the breakdown of exogenous pigments (results not shown).

Aging experiments were performed to see if the bleaching activity was also associated with supernatant fractions isolated from mature seedlings. Figure 8 shows that there was a progressive loss of bleaching activity with increasing plant age. Supernatants isolated on different days were stored at -80°C and assayed in the same experiment using the customary pellet from 20 h light-exposed seedlings as the source of pigments. Data are expressed per mg of supernatant protein in each case; storage of supernatant fractions at -80°C did not cause any loss of bleaching activity (results not shown). The recovery of protein in the supernatant decreased with aging (control, 5.2 mg/ml; 1 day, 4.8; 3 days, 4.0; 7 days, 2.8). Therefore, with time, 1 mg of supernatant protein represented a progressively greater fraction of biological material on a fresh-weight basis. If the data in Figure 8 were expressed as decrease in absorbance/ μ l

Table 3 Effect of fatty acids on pigment bleaching activities associated with (a) exogenous lipoxigenase and (b) endogenous supernatant

For (a), incubations contained 0.34 mg of pellet protein. For (b), incubations contained 0.62 mg of pellet and 2.9 mg of supernatant protein. All incubations contained 1 mM TX-100, except the last line in (a). Lipoxigenase was added at 2000 units/ml; fatty acids (in ethanol) were added to a concentration of 1.4 mM. Bleaching activity was determined from 30 min minus zero-time absorbance changes at 672 nm.

System	Bleaching activity	
	($10^3 \times$ Decrease in A_{672}/min)	(% of control)
(a) Pellet + lipoxigenase	3.1 ± 0.2	100
Pellet + lipoxigenase + LNA	10.3 ± 0.3	332
Pellet + lipoxigenase + LLA	9.8 ± 0.2	316
Pellet + lipoxigenase (without TX-100)	3.8 ± 0.2	123
(b) Pellet + supernatant	18.8 ± 0.5	100
Pellet + supernatant + LNA	5.1 ± 0.2	27
Pellet + supernatant	20.5 ± 0.3	100
Pellet + supernatant + LLA	13.1 ± 0.2	64
Pellet + supernatant	22.1 ± 0.4	100
Pellet + supernatant + oleate	12.8 ± 0.3	58

of supernatant added, the difference between the various ages would be even more striking. However, with age the cotyledons become harder to grind, which reduces the chloroplast recovery. We therefore preferred to express our data per mg of supernatant protein.

Comparison of bleaching by the chloroplast supernatant and by exogenous lipoxigenase

Lipoxigenase has long been known to cause pigment breakdown (Holden, 1965) in the presence of LLA or LNA. This enzyme has also been reported in chloroplast fractions from developing wheat (*Triticum aestivum*) tissue (Douillard, 1981; Douillard and Bergeron, 1979). The lipoxigenase system was compared with the endogenous system.

In the presence of soybean (*Glycine max*) lipoxigenase, thylakoid pigments were likewise degraded. LNA or LLA were required for significant lipoxigenase-induced bleaching (Table 3a). The lipoxigenase bleaching exhibited no requirement for TX-100, which, on the contrary, caused a slight inhibition (23.1 %) at 1 mM concentration (Table 3a).

On the other hand, if LNA, LLA or oleate were added to the endogenous supernatant system, inhibition of bleaching was observed (Table 3b). Addition of lipoxigenase to the endogenous system had no effect on the observed pigment destruction and, moreover, lipoxigenase did not counteract the inhibition of the endogenous system caused by unsaturated fatty acids (results not shown).

If the absorbance was monitored in the near-u.v. region, increases were observed at about 302–312 nm for both the endogenous and lipoxigenase-related systems (results not shown). The significance of these absorbance changes was not determined.

A range of inhibitors were tested on the endogenous bleaching system (Table 4). KCN was slightly inhibitory. The metal-ion chelator DIECA gave good inhibition, but the more polar desferal mesylate was ineffective. The very polar *o*-diphenol 4,5-dihydroxy-1,3-benzenedisulphonic acid was only weakly inhibitory, and so was resorcinol, an *m*-diphenol which cannot be

Table 4 Effect of various inhibitors on the endogenous pigment-bleaching system

This Table is compiled from a number of different experiments. Various inhibitors were tested on the reconstituted chloroplast pigment-bleaching system. All incubations contained chloroplast pellet, supernatant, 1 mM TX-100 and the respective inhibitor at the indicated concentration.

Inhibitor	Bleaching activity (% of control)	Reference
KCN (1 mM)	83.3	Lamberg and Legge (1949)
DIECA (1 mM)	46.2	Chereskin et al. (1982)
Desferal mesylate (1 mM)	105.0	Walker et al. (1991)
Methylene Blue (20 μ M)	75.1	
Methyl Viologen (20 μ M)	100.0	Chereskin et al. (1982)
PMS (20 μ M)	20.6	
Resorcinol (1 mM)	75.6	Huff (1982)
4,5-Dihydroxy-1,3-benzenedisulphonic acid (1 mM)	73.2	McRae and Thompson (1983)
Quinol		
100 μ M	45.9	Huff (1982);
1 mM	3.1	Afanas'ev (1989)
Benzoquinone		
100 μ M	24.3	Afanas'ev (1989)
1 mM	0	
Hydroxylamine (2 mM)	92.9	Afanas'ev (1989)
SOD		
500 units/ml	103.4	
2000 units/ml	108.3	Afanas'ev (1989); Elstner (1987)
Catalase		
10 units/ml	103.5	
1000 units/ml	100.1	Elstner (1987)

oxidized to a quinone by simple removal of the two phenolic hydrogens. On the other hand, the *p*-diphenol quinol, which is easily oxidized to benzoquinone, was a strong inhibitor, and benzoquinone itself was even stronger. Artificial electron acceptors (Methylene Blue, Methyl Viologen and phenazine methosulphate, which strongly inhibit the oxidative cyclase system (Chereskin et al., 1982), were tested on the bleaching reaction. Of these, the latter gave a strong inhibition, Methylene Blue was slightly inhibitory, but Methyl Viologen was ineffective. SOD and catalase had no effect on the endogenous system, indicating that probably free $O_2^{\cdot -}$ and free H_2O_2 are not involved in pigment breakdown. Hydroxylamine had essentially no effect, which confirmed the lack of inhibition observed with SOD, since hydroxylamine scavenges $O_2^{\cdot -}$, as it is rapidly oxidized to NO_2^- (Afanas'ev, 1989; Elstner, 1987).

DISCUSSION

We have observed an endogenous chloroplast pigment degradation which is not photochemical and which requires molecular oxygen. Up to this point, our experimental measurements have been spectrophotometric, and, therefore, limited to pigment changes. It is conceivable, however, that this phenomenon involves also a massive destruction of colourless membrane constituents (proteins, complex lipids, etc.), as well as actual pigment degradation. This pigment breakdown system decreases in activity as the chloroplasts mature and is, therefore, in agreement with the observations of Stobart and Hendry (1984) and Hendry and Stobart (1986) on greening wheat and barley (*Hordeum vulgare*) shoots *in vivo*.

Experimental research on chlorophyll breakdown in chloroplasts isolated from mature or senescing tissues has led to the

consideration of three non-light-dependent processes that may contribute to pigment degradation *in vivo* (Hendry et al., 1987; Brown et al., 1991). These are (a) pigment degradation coupled to the oxidation of unsaturated fatty acids by the enzyme lipoxygenase (lipoxygenase) (Holden, 1965), (b) pigment oxidation by H_2O_2 catalysed by a peroxidase in the presence of a monophenol or an *m*-diphenol (Matile, 1980; Huff, 1981; Martinoia et al., 1982) and (c) an O_2 -requiring system ('chlorophyll oxidase') quite distinct from the aforementioned lipoxygenase and peroxidase, which is stimulated by mono- and poly-unsaturated fatty acids and which converts chlorophyll into its C-10 hydroxy derivative (Fischer formula) (Martinoia et al., 1982; Lüthy et al., 1984; Schoch et al., 1984).

All three processes may well participate in the seasonal breakdown of chloroplast pigments that occurs in deciduous species in the autumn. The system we have described here appears to be different from these three processes. To begin with it was isolated from developing, rather than senescing, chloroplasts. Moreover, it does not require, and in fact is inhibited by, unsaturated fatty acids (Table 3); it is not stimulated, but rather is slightly inhibited, by 1 mM resorcinol (Table 4), and it is not inhibited by catalase at 1000 units/ml (Table 4). For these reasons we do not see how our results can be reconciled with either the lipoxygenase-dependent system, the peroxidase-dependent system or the 'chlorophyll-oxidase' system.

The most striking property of our own pigment breakdown system is the ease with which it can be separated from chloroplast membranes by simple osmotic shock. Even though our normal preparation of the active supernatant contains small membrane fragments (which do not interfere with the spectrophotometric measurements), ultracentrifugation yields a perfectly clear fluid without any loss of activity. Occasionally, however, we have noticed that the thylakoid pellet, which we use routinely as a source of pigments, possessed some enzymic activity without the addition of supernatant. In that case, the pellet was washed repeatedly with buffer B to remove this residual activity.

The stromal supernatant appears to lose activity on a protein basis as the dark-grown partially greened seedlings are placed in the greenhouse under natural photoperiodic conditions and are allowed to mature there for 1–7 days. Preliminary results suggest that cyclase activity also decreases with age in a manner analogous to what we have observed here for the endogenous pigment breakdown. However, this point has not been re-examined systematically. The correlation between these two activities as the seedlings are exposed to photoperiodic conditions is an interesting question that needs to be investigated.

The survey of potential inhibitors generated some interesting preliminary findings (Table 4). Those compounds that are potentially able to react with the reduced oxygen forms, $O_2^{\cdot -}$ and H_2O_2 , if they are also lipophilic enough to reach a hydrophobic pocket in a complex protein (like benzoquinone and quinol), were strong inhibitors of the endogenous bleaching system. However, if they are large or polar and, therefore, unable to reach a hydrophobic site (like dihydroxybenzenedisulphonic acid, hydroxylamine, catalase, and SOD), they were not strongly inhibitory. Similarly, DIECA, a lipophilic chelating agent, and CN^- , a haemoprotein-complexing agent which, at pH 7.7, is largely un-ionized and able to diffuse into a hydrophobic environment, were both inhibitory. Additional research will be needed to test the significance of these initial observations.

Developing chloroplasts possess numerous O_2 -requiring systems that are involved in the synthesis of essential constituents of the photosynthetic membranes, including coproporphyrinogen oxidase (Beale & Weinstein, 1990), protoporphyrinogen oxidase (Beale & Weinstein, 1990), and the oxidative cyclization of

MgProtoMe to DVPchl_a (Chereskin & Castelfranco, 1982; Walker et al., 1989). These processes are part of the biosynthesis of chlorophyll *a*. The synthesis of chlorophyll *b* from chlorophyll *a* (or chlorophyllide *a*) is also most probably an O₂-requiring process (Schneegurt and Beale, 1992).

Moreover, the conversion of phytoene into lycopene, and the conversion of carotenoid precursors into various xanthophylls (Kleinig, 1989) are also plastidic O₂-requiring processes leading to the formation of thylakoid carotenoids. Finally, the synthesis of the unsaturated complex lipids of the thylakoid membranes requires a series of fatty acid (or fatty acyl derivative) desaturases, which probably require O₂ and which may occur in the developing chloroplasts (Jaworski, 1987). Therefore it is not surprising that anaerobiosis blocks the normal development of chloroplasts (Vander Zee and Kennedy, 1982).

We speculate that at least some of these anabolic processes may involve a common mechanism of O₂ activation, probably through some localized oxygen free radical. In the presence of TX-100, the activated intermediate may become free to attack membrane constituents. The inverse relationship observed between the cyclase and the pigment breakdown activities (Figures 3 and 4) is consistent with this hypothesis. Thus the destruction of plastid pigments would be a symptom of generic metabolic disruption, and we predict that TX-100 may interfere with other O₂-requiring reactions involved in the synthesis of other thylakoid constituents.

Most recently, the study of chlorophyll breakdown during leaf senescence has been crowned by considerable success when P. Matile and his associates described a number of early breakdown products and identified conclusively one of them (Kräutler et al., 1991, 1992). On the other hand, in the present study we have only described chloroplast pigment breakdown in general without analysing the sequence of disappearance of the individual pigments or the appearance of putative degradation products. Further research will be needed to ascertain whether our findings with developing chloroplasts are consistent with the extensive research of P. Matile's group on senescing chloroplasts.

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