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Chlorophyll: a symptom and a regulator of plastid development

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CONTENTS

SUMMARY

The metabolism of chlorophylls and related tetrapyrroles directly influences, and is influenced by, the proteins and cell structures with which they are associated. During net accumulation, de-greening and at the steady state, chlorophyll and its derivatives are important elements in the post-translational regulation of the expression of genes for chloroplast proteins. At the same time, they represent potential photodynamic hazards against which green cells need to have protective mechanisms. This review deals with genetic, chemical and environmental

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Abbreviations: ALA, 5-aminolaevulinate; CC, core complex; ELIP, early light-inducible protein; FCC, fluorescent chlorophyll catabolite; LHC, light-harvesting complex; LHCP, light-harvesting chlorophyll-binding protein; LSU, large subunit; NCC, non-fluorescent chlorophyll catabolite; Nfl, Norflurazon; PhaO, Phaeophorbide *a* oxygenase; PLB, prolamellar body; POR, NADPH-protochlorophyllide oxidoreductase; Pr/Pfr, red/far-red absorbing forms of phytochrome; PS, Photosystem; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit; WRL, weak red light.

perturbations of chlorophyll biosynthesis that impact on protein stability, membrane organization and susceptibility to photodamage. NADPH-protochlorophyllide oxidoreductase is considered in detail as a pigment–-protein regulating, and regulated by, chlorophyll metabolism. The question of the extent and significance of chlorophyll turnover at the steady state is addressed, with particular emphasis on the dynamics of the photosystem II reaction centre. The pathway of chlorophyll catabolism is described, along with its interrelationship with protein mobilization in chloroplast senescence. Finally, the structural basis of pigment–protein interaction and stability is examined, and the discussion ends by expressing some general thoughts about the control of protein lifetimes in the living cell.

Key words: Chlorophyll *a* and *b*, tetrapyrroles, chlorophyll–protein complexes, chlorophyll biosynthesis, turnover and catabolism, plastids.

I. INTRODUCTION

Chlorophyll is familiar. The light absorbed and reflected by green plant tissues closely matches the light sensitivities of the eyes of Old-World primates, including *Homo sapiens*. There is good evidence that the L–M (red–green) cone system has evolved through spectral tuning by leaf reflectance, a dominant feature of the visual environment (Osorio & Bossomaier, 1992). Its very familiarity makes it easy to overlook chlorophyll and to underestimate its significance. Of course, it is universally appreciated that chlorophyll is part of photosynthesis, intercepting light energy that ultimately powers the assimilation of CO_2. But chlorophyll is far from being merely a passive colouring agent. The message of this discussion is that it plays a crucial part in correctly building the photosynthetic apparatus and in the controlled deconstruction of plastids during senescence.

II. CHLOROPHYLL AND RELATED TETRAPYRROLES

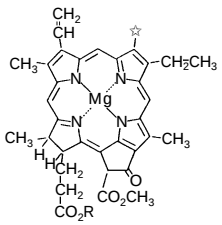
Figure 1 summarizes the chemical constitutions of chlorophylls *a* and *b* and related compounds found in green plants. Some features of these structures are relevant to the subject of the regulatory functions of macrocyclic tetrapyrroles. Chlorophylls and their proto- and phaeo- derivatives have more or less extensive conjugated double-bond systems and thus absorb strongly at visible (particularly red and blue) as well as ultraviolet A wavelengths. Absorption of light quanta by chlorophyll and related tetrapyrroles results in excitation to the singlet state (^{1}Chl). If the energy of absorbed quanta cannot be dissipated through photosynthetic electron transport or some other quenching mechanism, the molecule can undergo spin-inversion intersystem-crossing to produce the much longer-lived triplet (^{3}Chl) state. Triplet-state tetrapyrroles are highly active photosensitizers. Photodynamic damage can be propagated in cells either directly by reaction between ^{3}Chl and substrates such as lipid fatty acids to produce a free radical cascade (type I mechanism) or by a type II mechanism in which reaction with O_2 produces toxic singlet oxygen (Spikes & Bommer, 1991).

Chlorophyll, its biosynthetic precursors and its breakdown products are potentially destructive when illuminated. This poses special problems for pigment metabolism during net assembly and dismantling of the photosynthetic apparatus, as well as at the steady state. In the viable green cell, the structural role of chlorophyll and the need to hold its photodynamic tendencies in check are inseparable.

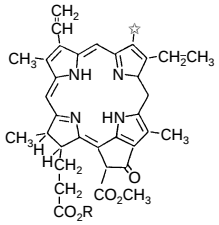
III. CHLOROPHYLL–PROTEIN COMPLEXES

1. *Light-harvesting and core complexes*

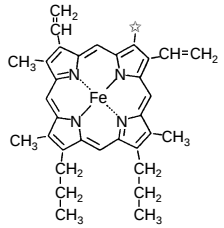
Free chlorophyll is readily isolated from green tissues by lipophilic solvents. Gentler extraction procedures, using subcellular fractionation followed by incubation of green membranes with surfactants, remove pigments as complexes with specific proteins (Ogawa, Obata & Shibata, 1966; Thornber, Smith &



R = H Chlorophyllide
R = phytol Chlorophyll
☆ = CH_3 Chlorophyll(ide) *a*
☆ = CHO Chlorophyll(ide) *b*



R = H Phaeophorbide
R = phytol Phaeophytin
☆ = CH_3 Phaeo *a*
☆ = CHO Phaeo *b*



Haem

Figure 1. Structures of chlorophyll and related tetrapyrroles.

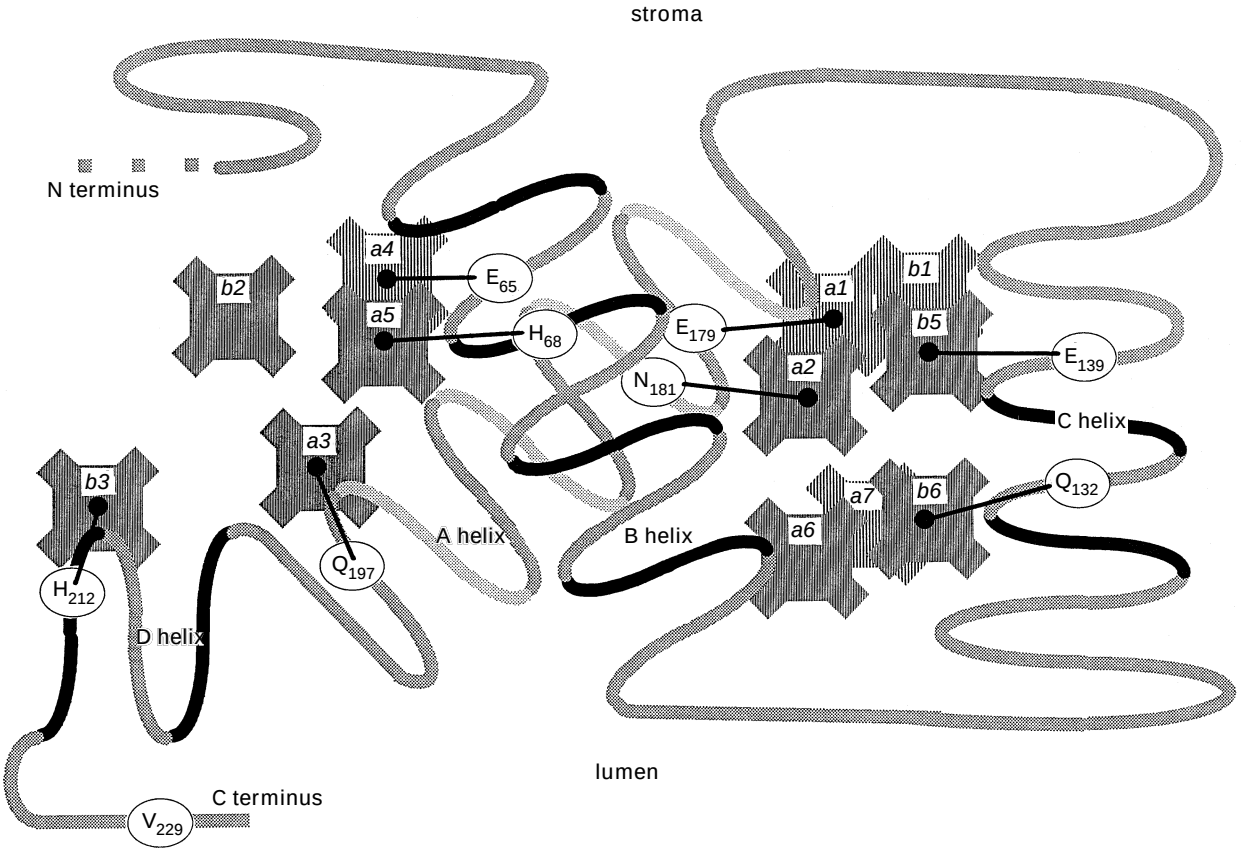


Figure 2. Schematic diagram of LHCP II in relation to chlorophylls and orientation in the thylakoid membrane. Significant amino acids are indicated by single-letter codes and sequential positions in the polypeptide. Chlorophylls are labelled *a1–a7* and *b1–b6*. After Kühlbrandt, Wang & Fujiyoshi (1994).

Bailey, 1966). The best modern procedures yield a dozen or more separable complexes and little or no free pigment. For example Photosystem (PS) I from barley has been resolved by electrophoresis into a core complex (CC I) and at least two light-harvesting chlorophyll–protein complexes (LHC Ia, Ib). CC I accounts for *c*. 20% of the total chlorophyll of the membrane and is composed of a major polypeptide of *c*. 58 kDa and chlorophyll *a* but not *b*. The LHC I complexes are made of chlorophylls *a* and *b* with apoproteins in the range 20–25 kDa (Dreyfuss & Thornber, 1994). Barley PS II gives a more complicated pattern, with at least three CC II and four LHC II complexes resolved by electrophoresis. LHC IIb is the major pigment–protein complex in the membrane, accounting for 40% of the total chlorophyll and is composed of apoproteins of 25–30 kDa and chlorophylls *a* and *b* (Peter & Thornber, 1991).

2. *Resolution of the molecular structure of LHC*

The nature of the association between pigments and proteins has been established in atomic detail for several complexes of prokaryotic origin (Papiz *et al*., 1996). Of the pigment proteins of angiosperms, the best characterized is the light-harvesting complex of angiosperm PS II, which has been resolved to 0·34 nm by Kühlbrandt, Wang & Fujiyoshi (1994). On gentle isolation from thylakoids, LHC II is obtained as a trimer. Each monomer comprises a single LHCP molecule (*c*. 230 amino acids, relative molecular mass 25 kDa), 12–15 chlorophylls (*a* and *b* in approx. equal molar ratio) and two carotenoids (usually lutein – but see below). Based on hydropathy plots of the derived amino acid sequence of LHCP, it had been anticipated that the protein has three membrane-spanning helices; and studies of membrane orientation had shown that the C and N termini of the polypeptide are luminal and stromal respectively. Electron crystallography confirmed these features of LHC II structure and enabled 12 chlorophyll molecules and the two carotenoids to be accurately positioned within the molecular structure of the monomer unit (Kühlbrandt *et al*., 1994).

The schematic in Figure 2 shows an N terminal segment of 54 amino acids leading to the B membrane-spanning helix (amino acids 55–89), a non-helical luminal loop, the C helix from residue 123 to 143, a stromal loop, then the A helix (170–199) and finally the C-terminal segment on the lumen side, including a small run of helix from 205 to 214. The two-fold symmetry that gives a distinctive X-shape to spans A and B is braced by two luteins, one running from about residue 197 (luminal end of helix A) to 160 (stromal loop between helices A and C) and

the other from about 48 (N terminal stromal segment) to around 100 (luminal loop between helices B and C).

Distinguishing chlorophyll *a* from *b* is beyond the level of resolution of electron crystallography. Nevertheless, spectroscopic and other evidence allows each of the twelve chlorophyll molecules within the crystal structure tentatively to be assigned an identity. These are shown in Figure 2, together with their presumptive ligands within the helices. Histidine (H) is the commonest ligand in other tetrapyrrole-polypeptide complexes; here only two chlorophylls (*a5* and *b3*) are coordinated in this way. Glutamine (Q–*a3, b6*), glutamate (E–*a1, a4, b5*) and asparagine (N–*a2*) are other identifiable ligands (Figure 2). The distances between the individual chlorophylls and between chlorophylls and carotenoids are consistent with known energy transfer rates.

The functions of the lutein molecules are of interest. A structural role is suggested by observations that *in vitro* assembly of the complex from the separate components is absolutely dependent on the presence of stoichiometric amounts of the carotenoid (Plumley & Schmidt, 1987). A light-harvesting function is considered less likely, but special significance is attached to the efficiency with which carotenoids quench chlorophyll triplets. Although lutein normally does these important jobs within LHC II, it is not essential for photosynthesis. Pogson *et al*. (1996) isolated lutein-less mutants of *Arabidopsis* that have perfectly normal levels of chlorophylls and apparently fully functional light-harvesting structures. Lutein is replaced by violaxanthin in these mutants, though this carotenoid is less effective than lutein when used for *in vitro* assembly (Plumley & Schmidt, 1987). The part played by chlorophylls in assembling LHC II is discussed in Section XI.

IV. CHLOROPHYLL BIOSYNTHESIS

1. *The C5 pathway*

Early in mesophyll cell development, chlorophyll–protein complexes are put together. Chlorophyll biosynthesis has a direct influence on expression of genes for plastid proteins, particularly at the post-transcriptional level. An outline of the C5 pathway of tetrapyrrole synthesis in green plants is shown in Figure 3.

At all stages of leaf and plastid development, from etiolated (von Wettstein, Gough & Kannangara, 1995) through to senescent (Hukmani & Tripathy, 1994), externally-supplied 5-aminolaevulinate (ALA) is metabolized in the dark to protochlorophyllides, the immediate precursors of the light-requiring step at NADPH-protochlorophyllide oxidoreductase (POR). This tells us that the section of the biosynthetic pathway between ALA dehydratase and POR is probably constitutive. Because protochlorophyllides do not normally build up in dark-incubated tissue, either ALA supply must be limiting or products downstream of ALA are diverted to other fates. Studies with the *tigrina* series of barley mutants reveal the existence of at least four genes concerned with regulating the flow of metabolites between ALA and protochlorophyllide. Feedback inhibition is a possibility, since both haem and divinyl-protochlorophyllide have been observed to inhibit ALA formation *in vitro* (Castelfranco & Beale, 1983). Genetic manipulation has to some degree simulated in light-grown plants the suppressed state of the ALA to protochlorophyllide pathway characteristic of etiolated tissue: transgenic tobacco plants expressing antisense genes for the ALA-forming enzymes glutamate-tRNA synthase (Andersen, 1992) and glutamate-1-semialdehyde aminotransferase (Höfgen *et al*., 1994) are pale or variegated. Light causes elevated steady-state levels of mRNAs encoding some enzymes in the tetrapyrrole biosynthetic pathway, for example ferrochelatase (Smith *et al*., 1994), but depresses others such as the Mg chelatase component *OLIVE* (Hudson *et al*., 1993) and POR (see Section V). Another facet of the regulation of chlorophyll biosynthesis that merits study in higher plants was described by Johanningmeier (1988), who found evidence that intermediates in the pathway influence the expression of nuclear genes in *Chlamydomonas*. It has been proposed that tetrapyrroles are important elements in the signalling mechanism that co-ordinates nuclear gene expression and plastid differentiation (Reinbothe *et al*., 1996)

2. *Degradation of C5 pathway intermediates*

It is reasonable to conclude that substrate-level regulation by feedback loops is essential for matching the supply of photodynamic precursors and end-products to the capacity of quenching mechanisms to conduct them safely into stable complexes. Implicit in such a scheme is a requirement for intermediates upstream of an inhibited step to be redirected into catabolism, but the biochemistry of these branch-lines in the metabolic network is rather poorly defined. Oxidative enzymic activities able to bleach chlorophyll and precursors have been detected inside and outside immature plastids and chloroplasts (Hougen, Meller & Gassman; 1982; Lüthy *et al*., 1984; Whyte & Castelfranco, 1993; Jacobs & Jacobs, 1993; Shioi *et al*., 1995; see Fig. 4). We may speculate that they represent built-in escape routes that render potentially phototoxic porphyrin precursors harmless. Some measurements of the turnover of chlorophyll and precursors in etiolated and greening tissues suggest that these catabolic systems are surprisingly active (Stobart & Hendry, 1984; Hendry & Stobart, 1986).

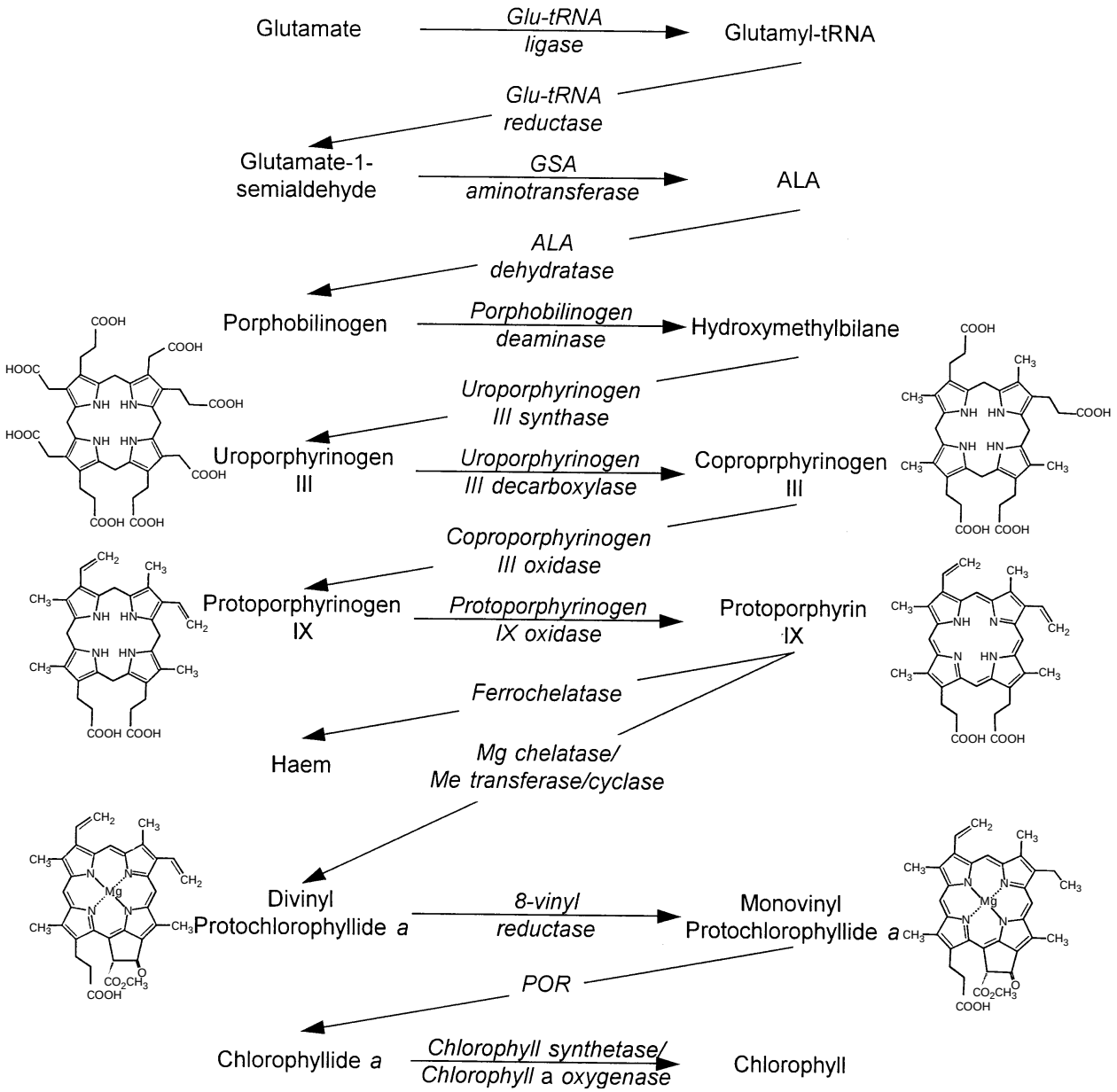


Figure 3. The C5 pathway of chlorophyll biosynthesis. Structures of photodynamic intermediates are shown, as is the origin of haem.

3. *Genetic perturbation of chlorophyll biosynthesis*

The regulatory capacities of the tetrapyrrole biosynthetic network are limited, however, and may be easily overridden chemically or genetically. The tetrapyrrole intermediates accumulated when ALA is fed in the dark are inadequately served by the normal *in vivo* quenching mechanisms so that when the tissue is illuminated, there is often fatal photodamage. Mock *et al.* (1995) and Kruse, Mock & Grimm (1995) rendered tobacco plants highly photosensitive by means of antisense down-regulation of uroporphyrinogen decarboxylase and coproporphyrinogen oxidase, respectively. Chlorophyll *b*-less mutants are known in a number of plant species. Recently it has been shown that many of these do not represent biochemical lesions in the chlorophyll *a*/*b* interconversion but rather have leaky mutations of earlier steps in the common pathway (Falbel & Staehelin, 1996). A threshold level of chlorophyll *a* seems to be required before chlorophyll *b* biosynthesis can commence. Thus less extreme disturbance of the pathway than dark-feeding precursors or antisense knockout is expressed in *b*-less mutants by suppressing chlorophyll *b* synthesis and the associated assembly of light-harvesting complexes.

4. *Early light-inducible proteins*

Within a few hours of exposing etiolated seedlings to light, distinctive mRNAs appear and increase to maximal levels (Meyer & Kloppstech, 1984; Grimm & Kloppstech, 1987). These messages encode early light-inducible proteins (ELIPs) which become

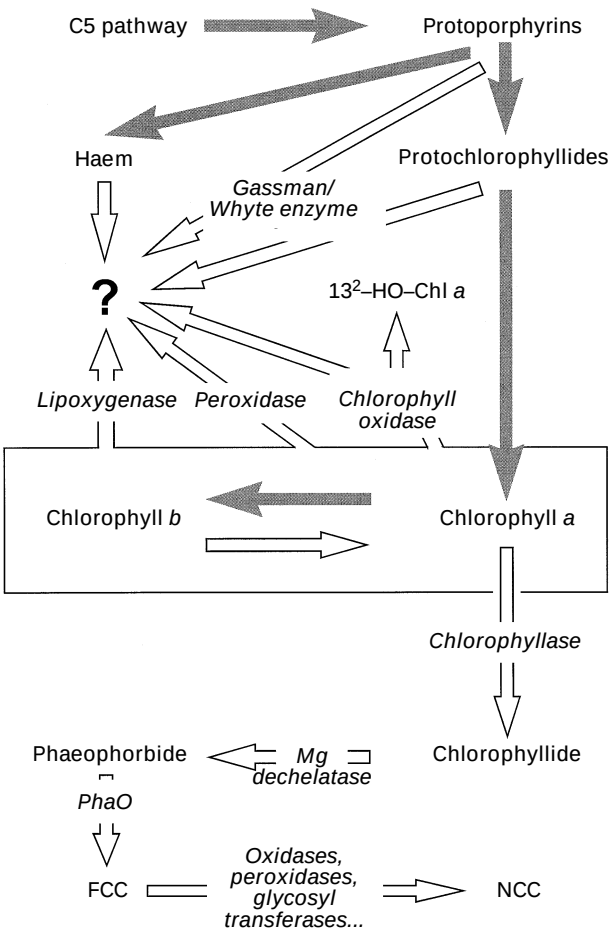


Figure 4. Degradation of chlorophyll and related compounds in green cells of angiosperms. Biosynthesis is represented by filled arrows, breakdown by open arrows. The products of (per)oxidation are unidentified, except for 13²-HO-chlorophyll *a*, shown by Schoch *et al.* (1984) to be amongst the reaction products of chlorophyll oxidase.

integrated into the thylakoid membrane. It is now known that ELIPs are induced at all stages of leaf development, even into senescence, in response to high light (photoinhibitory) stress (Potter & Kloppstech, 1993; Humbeck, Kloppstech & Krupinska, 1994). Gene sequence comparisons establish ELIPs to be members of the LHCP multigene families (Green & Pichersky, 1994). That ELIPs are likely to be pigment–proteins may be inferred not only from their LHC-like structures (Green & Kühlbrandt, 1995) but also from their homology with algal carotenoid-binding proteins (Levy *et al.*, 1993). ELIPs are considered to be important factors in controlling photodynamic damage when the intensity of illumination threatens to overrun the quenching capacity of the photosynthetic apparatus. By binding photoconvertible xanthophylls, they can act as hyper-stable LHCs under light stress (Krol *et al.*, 1995). ELIP mRNA is short-lived and, during recovery from light-stress, the protein is rapidly degraded (Adamska, Kloppstech & Ohad, 1993), perhaps by a thylakoid-associated serine protease (Adamska *et al.*, 1996). There is no evidence either way, but one may speculate that, like other pigment-associated proteins, alteration in the nature of the association between apoprotein and chromophore may trigger proteolysis.

V. NADPH-PROTOCHLOROPHYLLIDE OXIDOREDUCTASE

1. *General features of POR regulation*

Each of the steps in chlorophyll biosynthesis in which macrocyclic tetrapyrroles are substrates or products has implications relevant to the subject of the present review, but one invites particular attention. POR represents an interesting model for chlorophyll–proteins in general, illustrating two major regulatory themes: light-mediated changes in transcription; and post-translational control via differential proteolytic susceptibility. POR is an important enzyme in the control of chloroplast assembly. It is the point at which the chlorophyll biosynthetic pathway is light-dependent (Griffiths, 1978). The enzyme uses light as a kind of substrate. It is abundant in dark-grown tissues, as is its mRNA, but on exposure to light, amounts decrease (Mapleston & Griffiths, 1980; Forreiter *et al.*, 1990). POR expression is modulated by the phytochrome system at the level of repression of nuclear gene transcription (Mösinger *et al.*, 1985). It is regulated post-translationally via turnover, resulting in the decline of the protein in the light (Kay & Griffiths, 1983). In the absence of light, POR exists as a stable enzyme-substrate complex between apoprotein, NADPH and protochlorophyllide (Oliver & Griffiths, 1982). On illumination, there is conversion to an enzyme-product complex which is highly susceptible to proteolytic attack (Reinbothe *et al.*, 1995*c*).

2. *PORA and PORB*

The POR story just presented is now known to apply to PORA, one of two light-dependent POR enzymes found in angiosperms. The highly photoregulated PORA characteristic of greening etioplasts is present at vanishingly small levels in green tissues, and yet there is abundant evidence that such tissues can photoreduce protochlorophyllide. The enzyme responsible is referred to as PORB. Holtdorf *et al.* (1995) have described just such a form of POR: it is closely related in protein and gene structure to PORA but is not expressed in a light- or phytochrome-sensitive manner. This form of POR also differs from PORA in the mechanism whereby its precursor is imported into the plastid from the site of synthesis in the cytosol.

PORA can be expressed as the unprocessed precursor pPORA in *E coli* transformed with cDNA encoding the barley enzyme (Schulz *et al.*, 1989).

Table 1. *Abundances of plastid types in primary leaves of wheat treated with water, 0·1 mM GA_3 and/or 1 mM gabaculine in darkness for up to 48 h after germination (data of Younis, Ryberg & Sundqvist, 1995)*

Hours	Treatment	Amyloplasts (%)	Proplastids (%)	Young + mature etioplasts (%)
36	Control	49	18	33
	GA_3	15	7	78
	Gabaculine	84	13	3
	GA_3 + gabaculine	89	6	5
42	Control	26	12	61
	GA_3	2	0	98
	Gabaculine	72	16	12
	GA_3 + gabaculine	81	10	9
48	Control	6	6	88
	GA_3	0	4	96
	Gabaculine	42	0	58
	GA_3 + gabaculine	65	23	12

Radioactively-labelled pPORA is taken up by isolated etioplasts and processed to the mature size. Reinbothe *et al.* (1995*d*) showed that import is dependent on the availability of protochlorophyllide, with which pPORA associates before removal of the transit peptide. There is evidence that docking between pPORA and protochlorophyllide at the etioplast envelope is facilitated by chaperonin(s) and prevented by externally-supplied protochlorophyllide in a mutually antagonistic way. If pPORA is complexed with chlorophyllide it cannot be imported or processed (Reinbothe *et al.*, 1995*c*). Association with (proto)chlorophyllide clearly has profound influences on (p)PORA conformation, with implications for the enzyme's subcellular localization and metabolism. By contrast, PORB import to plastids does not require protochlorophyllide, though newly-imported PORB forms a stable ternary complex with protochlorophyllide and NADPH (Reinbothe *et al.*, 1995*b*).

3. *POR and plastid differentiation*

Etioplasts contain prolamellar bodies (PLBs), paracrystalline structures largely made of lipids and ordered arrays of PORA in its photosensitive ternary enzyme-substrate complex form. Illumination disperses the PLB as PORA is degraded and the plastid develops into a chloroplast. PORA has a structural role in PLB assembly, and the supply of protochlorophyllide is decisive for this role. For example, Table 1 presents data of Younis, Ryberg & Sundqvist (1995) on the proportions of different plastid types in dark-grown wheat seedlings over a period of intense etioplast differentiation. Between 36 h and 48 h of seedling development etioplasts increased from 33 to 88 % of the total plastid population.

Gabaculine is an inhibitor of tetrapyrrole biosynthesis in plants. In etiolated wheat tissue treated for 42 h with 1 mM gabaculine, synthesis of non-phototransformable protochlorophyllide was decreased by *c.* 80 %. Etioplast differentiation was correspondingly slowed, so that even at 48 h only just over half the plastids were etioplasts (Table 1).

Early processes in chloroplast differentiation have also been studied along the age-gradient of expanding grass leaves. Here the youngest cells at the base of the lamina are enclosed by the sheaths of previous leaves and contain organelles with characteristics intermediate between etioplasts and proplastids. Chloroplasts become fully differentiated as cells emerge from the sheath into full light. The expression pattern of PORA along the age gradient of cells in the fourth leaf of *Lolium temulentum* seedlings is similar to that observed during greening of etiolated whole leaves (Davies *et al.*, 1989; Ougham & Davies, 1990). The proplastids of young leaf cells of *Lolium* treated with gabaculine lack any internal membrane structure, and assembly of the thylakoids in maturing chloroplasts is severely disrupted. This is accompanied by a marked inhibition of chlorophyll and LHCP II formation (Davies *et al.*, 1990*a*). Schünmann, Ougham & Turk (1994*b*) and Schünmann & Ougham (1996) also exploited the developmental gradient along the expanding leaf in a study of *slender*, a barley overgrowth mutant with much decreased sensitivity to low temperature constraints on cell extension (Schünmann, Harrison & Ougham, 1994*a*). They isolated by differential screening a cDNA corresponding to an mRNA that is abundant in young expanding tissue of wild-type leaves but very much less so in the mutant. Sequencing revealed this cDNA to be identical with PORA. Expression of POR along a gradient of cell age in barley leaves was similar to that observed in *L. temulentum*. Altered expression in *slender* implies that the growth of the cell within which plastids are differentiating also

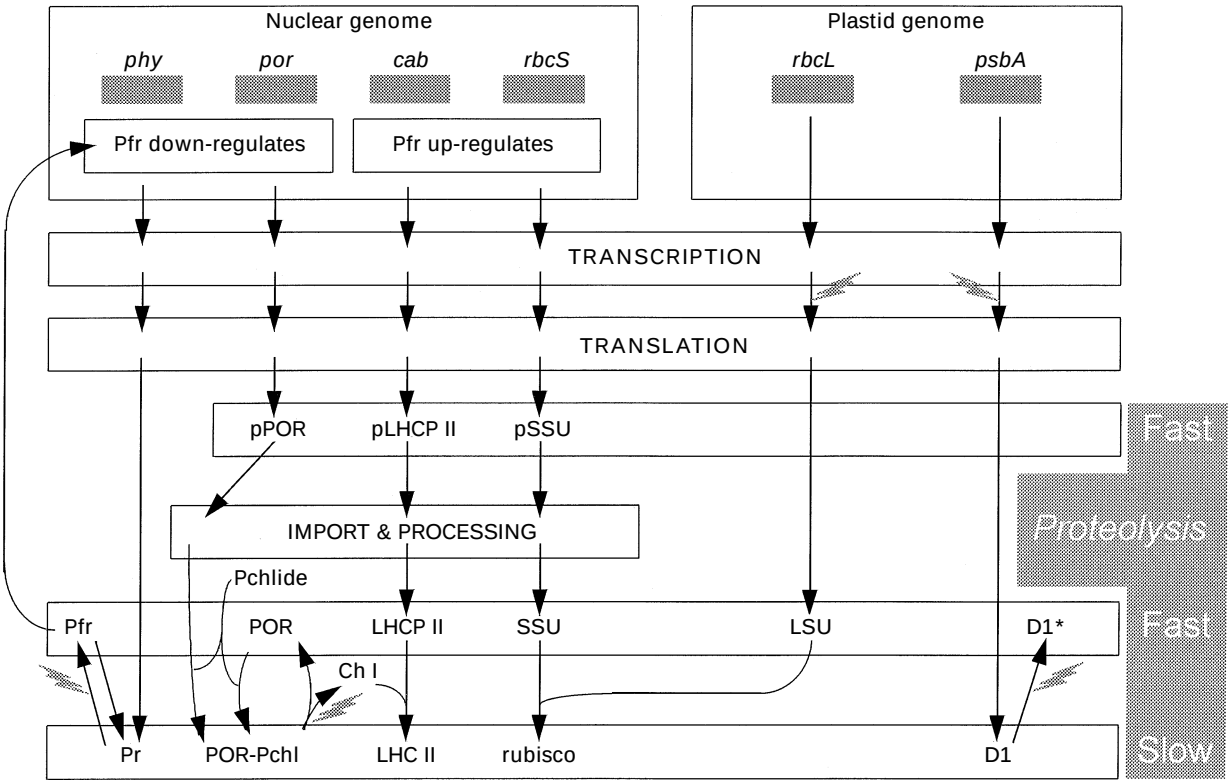


Figure 5. General features of transcriptional and post-transcriptional regulation of gene expression for some important plastid proteins, emphasising post-translational protein:chlorophyll and protein:protein interactions. Light exerts its influence through the phytochrome Pr/Pfr system (encoded by nuclear gene *phy*) and also directly modifies the stability of POR (nuclear gene *por*) and the D1 protein of PS II (plastid gene *psbA*). Chlorophyll supply determines the lability of LHCP (nuclear gene *cab*). Stoichiometric association of the large (LSU) and small (SSU) subunits (genes *rbcL*, *rbcS*) is necessary for Rubisco function and survival. *Fast* and *Slow* refer to the susceptibility of different forms of each protein to proteolytic attack.

exerts a regulatory influence over POR, though the mechanism is not clear. The etiolated leaf stimulated to green by transfer from darkness to full illumination is a very convenient experimental subject that has revealed much about POR and its controls. Nevertheless, there are special features of POR during plastid differentiation in growing cells under less extreme light regimes that are better studied in these more 'natural' developmental systems.

An intriguing aspect of plastid differentiation raising further questions about POR metabolism concerns the reversal of senescence. In some plants, notably *Nicotiana*, the plastids of yellow leaves (gerontoplasts–see Section VIII) can be induced to redifferentiate into chloroplasts. We found that PORA is immunologically undetectable in mature green and senescent yellow leaves of intact *N. rustica* plants. But if the yellow leaf is stimulated to regreen by cutting off the shoot above it, treating with cytokinin and maintaining the plant in dim light, the resumption of chlorophyll biosynthesis is accompanied by strong induction of POR (H. Zavaleta, B. J. Thomas, I. M. Scott & H. Thomas, unpublished). This implies that POR expression, import, assembly and turnover systems, like those working in the greening etioplast, probably also operate during the gerontoplast-chloroplast transition.

4. *POR degradation*

The ternary complexes of both PORA and PORB with protochlorophyllide and NADPH are stable in the dark. By contrast, the POR-chlorophyllide association is susceptible to proteolysis. *In vitro* it is very difficult to dislodge chlorophyllide from PORA and so it might be that each POR molecule survives only one turn of the reaction cycle, making it a truly suicidal enzyme. On the other hand there is evidence from studies with mutants that PORA *in vivo* is not strictly a one-shot enzyme and that, during greening of etiolated tissue, a given molecule of PORA can go round the catalysis cycle several times before it is degraded (Reinbothe *et al.*, 1996). Proteolytic activities have been detected within etioplasts and proposed to be responsible for attacking susceptible forms of PORA (Hammp & De Filippis, 1980; Walker & Griffiths, 1986; Honda, Tanaka & Tsuji, 1994). A light-induced nuclear-encoded ATP-dependent pH 6·5 protease that increases to maximal activity as the etioplast-to-chloroplast transition reaches completion might be responsible for regulating PORB turnover (Reinbothe, Apel & Reinbothe, 1995*a*; Reinbothe *et al.*, 1995b). Although the mechanisms of suicidal light-dependent turnover of POR are becoming clearer, the reasons for such an

apparently inefficient and wasteful molecular lifestyle are less obvious. Photoactive chromophore-associated proteins often carry out high-risk jobs in the cell and may be designed to be disposable (the D1 protein is another example–see Section VII). It has been suggested that proteolytic fragments of POR have a function in conveying newly made chlorophyllide to the developing thylakoid membrane (Reinbothe *et al.*, 1996). In any case, notions of materials-efficiency and energy-efficiency do not necessary have the same significance for plants that they do for animals and other heterotrophs (Thomas, 1994).

5. *Chlorophyll synthesis in the dark*

There is reason to believe that forms of POR other than A and B exist in higher plants. Based on careful quantifications of pigments and patterns of radiolabelling in barley, *Tradescantia* and *Zostera*, Adamson and co-workers have produced persuasive evidence that angiosperms can synthesize chlorophyll in the dark (Adamson, Hiller & Vesk, 1980; Adamson, Packer & Gregory, 1985; Walmsley & Adamson, 1995). Light-independent chlorophyll biosynthesis is well established in green algae, pteridophytes and gymnosperms. In these plants the chloroplastic genes *chlL*, *chlN* and *chlB* (structurally unrelated to PORA/B) encode a protochlorophyllide reduction pathway that can work in darkness (Suzuki & Bauer, 1992; Li, Goldschmidt-Clermont & Timko, 1993). These genes have been lost from the angiosperm plastid genome. There are no reports of anything like them in the nuclear genome either, so it might be that light-independent chlorophyll synthesis in angiosperms occurs via yet another form of POR. At present, the regulatory role of pigment–protein interactions in these emerging areas of POR metabolism can only be guessed at.

6. *POR in relation to expression and turnover of other plastid proteins*

In plastid assembly we see two major regulatory themes: light-mediated changes in transcription; and post-translational control via differential proteolytic susceptibility. Figure 5 is a picture of plastid assembly looked at in this way, showing a direct association between POR metabolism and construction of light-harvesting and other chlorophyll–protein complexes.

VI. ASSEMBLING CHLOROPHYLL–PROTEINS

1. *Integration of LHCP*

The mechanism of chlorophyll–protein complex assembly has been much studied and it is not my intention to review this subject comprehensively, but instead to focus on the regulatory role of pigments. The abundance of LHC IIb makes it a favoured subject for investigation. Precursors of the LHC II apoproteins (pLHCP) when presented to isolated plastids are taken up by an energy-requiring, post-translational mechanism (Waegemann, Paulsen & Soll, 1990). Soon after uptake, LHCP can be detected in LHC IIb within the thylakoids. Experiments with mutagenized pLHCP (Kohorn & Tobin, 1987) and with LHCP fused with the Rubisco transit peptide (Lamppa, 1988) have established that mature LHCP contains all the necessary information for correct targetting and assembly within the chloroplast. Integration of apoproteins into the thylakoid membrane requires Mg-ATP and at least two chaperonin-like stroma factors (Payan & Cline, 1991). It is thought that chlorophyll (*b* in particular) is also necessary for stable integration (Kohorn & Auchincloss, 1991), and studies *in vitro* show that apoproteins and pigments in the correct ratio have a strong tendency towards self-assembly (Plumley & Schmidt, 1987; Paulsen, Finkenzeller & Kühlein, 1993). Initial integration of (p)LHCP occurs largely in the intergranal thylakoid membranes, followed by migration into the stacked region (Kohorn & Yakir, 1990).

2. *Role of chlorophyll in stabilizing nascent LHCP*

Many different lines of evidence show that light-harvesting proteins uncomplexed to chlorophyll are subject to fast degradation. For example, chlorophyll *b*-less mutants express genes for LHCs, and make LHC proteins, at essentially normal rates, but without chlorophyll *b* to form complexes with correct stoichiometries, the proteins are turned over rapidly (White & Green, 1987; Harrison, Nemson & Melis, 1993). Exposing etiolated tissue to intermittent light also disturbs the balance between protein and chlorophyll supply during complex assembly, resulting in differential turnover (Bennett, 1981; White & Green, 1988; Tanaka, Tanaka & Tsuji, 1992). Dahlin & Timko (1994) used an *in vitro* integration system to show the relationship between pigment availability and stability of apoproteins during assembly of complexes. When pea plants were grown in weak red light (WRL), their leaves contained *c.* 29% of the chlorophyll and 66% of the carotenoids of high-light leaves. In plants exposed to WRL plus the carotenoid synthesis inhibitor Norflurazon (Nfl), pigments were further decreased to 14% and 5% respectively. When pLHCP was incubated with thylakoids plus plastid lysate it was integrated into the membrane. Compared with high-light controls, with plastid preparations from WRL plants only *c.* 30% of the integrated protein was stable to treatment with the protease thermolysin, whilst for WRL + Nfl the figure was only 5%. Thus the resistance of newly-integrated apoprotein to

proteolysis is nicely in step with the chlorophyll status of the tissue from which the plastid preparation was made. Reciprocal incubations with lysates and membranes from different sources and parallel experiments with the Rieske FeS protein, which does not complex with pigments *in vivo*, supported the notion that chlorophyll regulates the integration of its associated proteins, except at abnormally low carotenoid levels.

Conversely, antisense transgenic tobacco plants in which LHCP II transcripts have been decreased to almost undetectable levels make essentially normal amounts of properly integrated and functional LHC (Flachmann & Kühlbrandt, 1995). All in all, the story is consistent: chlorophyll is necessary for stabilizing complexes against attack by proteases *in vivo* and its rate of supply sets the pace for the rate of appearance of functional, integrated light-harvesting and reaction-centre units.

VII. CHLOROPHYLL TURNOVER AT THE STEADY STATE

1. *Metabolism of labelled chlorophyll*

We have seen that the chlorophyll biosynthesis pathway seems to remain in place in leaf tissue that has completed net accumulation of the pigment and passed into the steady state, and even into early senescence. If the capacity to make chlorophyll is present at and beyond chlorophyll steady state in leaf cell development, is there a balancing degradative capacity too? In other words, does chlorophyll turn over at a significant rate? This question remains generally unresolved. One early radiolabelling study showed considerable differences between dicots and monocots (Perkins & Roberts,1983). Incorporation of precursor into chlorophyll was high in mature dicot leaves and negligible in comparable monocot tissue. Differences in radiolabelling between the chlorophylls of different complexes within the same thylakoid membrane have also been reported (Brown, Acker & Duranton, 1975).

2. *D1 protein turnover*

A process with which continuous replacement of chlorophyll might be expected to be associated is turnover of the D1 protein of PS II. D1 has a short half-life in light and a high rate of degradation is matched by an equally high rate of synthesis (Mattoo *et al*., 1984). Under photoinhibitory conditions, D1 degradation in isolated PS II particles is simultaneous with, or even anticipated by, destruction of the reaction centre chlorophyll P_{680} (Telfer *et al*., 1994). D1 may be regarded as a chlorophyll-binding protein and therefore might be expected to become susceptible to proteolysis when dissociated from the pigment. Chlorophyll is certainly required for stable integration of newly synthesized D1 (Mullet, Gamble-Klein & Klein, 1990). A study with radiolabelled ALA by Feierabend & Dehne (1996) provided evidence that D1 turnover is probably accompanied by chlorophyll turnover *in vivo*, under moderate light as well as under photoinhibitory conditions. Clearly the supply of new chlorophyll is met by the operation of the whole C5 pathway and not only from pools of immediate precursors or chlorophylls transferred from other complexes. Raskin, Fleminger & Marder (1995) pulse-labelled barley leaves with [^{14}C]ALA and found that, over the short term, the specific radioactivity of reaction centre chlorophyll was much lower than that of thylakoids as a whole or of a grana-enriched fraction. On the other hand during a cold chase following a ^{14}C feed, reaction centres lost label faster than did thylakoids and grana. The authors suggest that these data are consistent with the existence of different pools of newly synthesized chlorophyll with fast, slow or intermediate turnover kinetics, to which the different pigment–protein complexes within the membrane have access.

The degradative side of the chlorophyll turnover equation seems likely to be photodynamic. It almost certainly does not occur through the catabolic pathway that operates when green tissue turns yellow in senescence, since a mutant of *Festuca pratensis* in which one step of this pathway is disabled (Vicentini, Iten & Matile, 1995) turns over D1 in the light perfectly normally (Hilditch, Thomas & Rogers, 1986). Protein turnover in the core complex of PS II is not the same in photoinhibition as under non-photoinhibitory conditions; the process when photoinhibition is exerted through donor-side limitation is different from that of acceptor-side photoinhibition (Barber & Andersson, 1992). But a common feature of all modes of D1 turnover might be the dislodging of chlorophyll leaving a proteolytically-susceptible apoprotein.

3. *Sun–shade acclimation*

Leaf and chloroplast structural and photosynthetic characteristics are sensitive to the light intensity under which they develop. Thus leaves developing in shade have a higher ratio of light-harvesting (relatively chlorophyll *b*-rich) to reaction centre (*a*-rich) complexes than those exposed to brighter light (Anderson, Chow & Goodchild, 1988). Mature leaves are also capable of acclimation to decreased light levels. Hidema *et al*. (1991) showed that exposing fully-expanded leaves of rice to low irradiance strongly retarded the post-maturity loss of total chlorophyll during senescence while the ratio of *b* to *a* appreciably increased. Similar responses were observed in *Lolium temulentum* leaves (Mae *et al*., 1993). Hidema *et al*. (1992) confirmed that the diminished rate of chlorophyll loss and the increase

in *b* : *a* ratio in shaded rice leaves were associated with retention of LHC II; furthermore, ^{15}N-labelling revealed that LHC II protein turned over at a negligible rate under these conditions. The evidence for continued synthesis and breakdown of non-reaction-centre chlorophyll under such steady-state conditions is at best contradictory. In the absence of much *de novo* synthesis, an increase in the *b* : *a* ratio could be explained by conversion of chlorophyll *a* to *b* during acclimation to shade. It may be that this in turn is part of a metabolic cycle regulating the balance of *b* to *a*, and hence that of light-harvesting to reaction centre. The evidence for, and wider significance of, a reaction scheme running from chlorophyll *b* to *a* are discussed in Section IX 5.

4. *Phaeophorbide in PS II*

A minor but intriguing aspect of chlorophyll metabolism during assembly and turnover of the PS II reaction centre concerns phaeophorbide. Spectroscopic evidence has established that the D1/D2 complex is associated with phaeophorbide *a* as well as chlorophyll *a*. The origin and structural significance of this chlorophyll derivative (which represents no more than 0·1 % of total chlorophyll) is not very clear, but some experiments using high-sensitivity luminescence analyses of greening membranes and tissues (Ignatov & Litvin, 1994) point to the chlorophyllide product of POR as its immediate precursor. It was suggested that chlorophyllide to (presumably) phaeophorbide is catalysed by Mg chelatase running in reverse, and that the complete sequence on through phytylation involves several steps and is light-regulated. There was evidence that assembly of the functional PS II core complex proceeds via an association between D1, D2, chlorophyll *a* and phaeophorbide *a*, lacking P_{680}. Radiolabelling studies are also consistent with biosynthesis, rather than *in situ* chlorophyll dechelation, as the origin of phaeophytin (Raskin *et al*., 1995). Phaeophytin biosynthesis might turn out to be a significant feature of PS II turnover; if so, it will be important to understand how the process is regulated (Ignatov & Litvin, 1994).

VIII. THE PLASTID FAMILY TREE

We have seen that net assembly of complexes is characteristic of the differentiation of chloroplasts from etio-granal, pro-granal or pre-granal plastids. Equally dramatic changes occur in other parts of the plastid developmental network (Thomson & Whatley, 1980). Senescence is the period of chloroplast to gerontoplast transition. Not everyone likes the term gerontoplast (coined by Sitte, 1977), but I do because it emphasizes that maturity to senescence represents not a loss or deterioration but a *change* of function in the life of the leaf. The gerontoplast is not a dead organelle, it is a plastid with the defined and controlled task of salvaging material for use elsewhere. The visible symptom of senescence is yellowing, which tells us that chlorophyll is being removed from the thylakoid membrane. Is the dismantling of thylakoid complexes during yellowing the converse, inverse or reverse of assembly during the greening of expanding tissue?

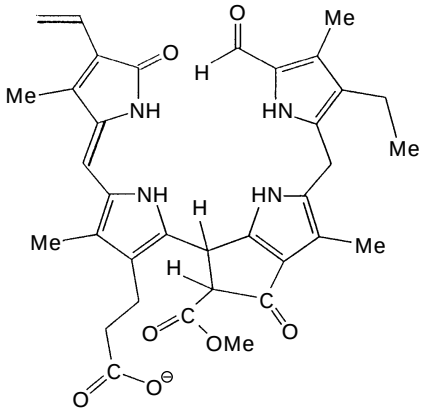
IX. THE PATHWAY OF CHLOROPHYLL CATABOLISM

1. *The first three reactions*

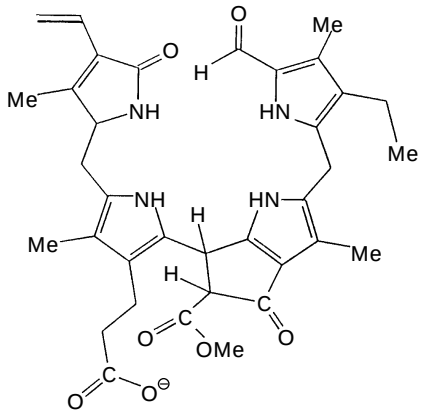
The breakdown of chlorophyll into phytol, Mg^{2+} and a primary cleavage product of the porphyrin moiety occurs in three consecutive reactions (Matile & Kräutler, 1995; Matile *et al*., 1996; Fig. 4). The first step in chlorophyll catabolism is removal of the phytol tail of chlorophyll *a* to produce chlorophyllide. Chlorophyllase, the enzyme that carries out this reaction, is associated with plastid membranes and is probably constitutive but latent throughout much of the development of green tissues (Amir-Shapira, Goldschmidt & Altman, 1987; Rodríguez, González & Linares, 1987; Brandis, Vainstein & Goldschmidt, 1996). Phytol seems to be quite stable during leaf senescence and persists largely in esterified form (Peisker *et al*., 1989). Although Mg falls out of chlorophyll all too easily *in vitro*, particularly if the pH is on the acid side, conditions in the cell do not ordinarily favour this and an activity called Mg dechelatase is required. Mg dechelatase was originally identified in green algae (Ziegler *et al*., 1988) and has been detected in higher plants by assaying the conversion of chlorophyllide into phaeophorbide or dechelation of chlorophyllin (Langmeier, Ginsburg & Matile, 1993; Vicentini *et al*., 1995; Shioi *et al*., 1996). Here again, the activity appears to be constitutive and latent. Phaeophorbide *a* oxygenase (PhaO), the third step, is most significant for the yellowing of senescent leaves because opening the porphyrin macrocycle is associated with the loss of green colour (Hörtensteiner, Vicentini & Matile, 1995).

2. *Phaeophorbide a oxygenase*

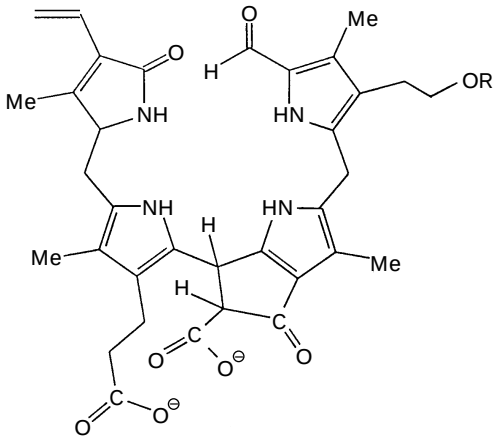
The first stable product of PhaO is a linear tetrapyrrole. The chemical structure of the fluorescent chlorophyll catabolite (FCC) made by the PhaO of rape cotyledons (FCC-2) has been determined (Fig. 6*b*). The reaction that produces FCC-2 requires O_2 and involves Fe which operates in a redox-cycle driven by reduced ferredoxin (Matile *et al*., 1996). PhaO is specific for phaeophorbide *a* as substrate; phaeophorbide *b* is a competitive inhibitor of FCC production *in vitro* (Hörtensteiner *et al*.,



(*a*) Reaction intermediate



(*b*) FCC-2



(*c*) NCCs

NCC-1 R = $COCH_2COO–$
NCC-2 R = $C_6H_{11}O_5$ (β-glucose)
NCC-3 R = H

Figure 6. Structures of chlorophyll catabolites from *Brassica napus*. (*a*) First detectable intermediate in the macrocycle-opening phaeophorbide *a* oxidase reaction. (*b*) Fluorescent product of the PhaO reaction. (*c*) Three non-fluorescent terminal catabolites (Matile *et al*., 1996; Mühlecker & Kräutler, 1996).

1995). The production of primary FCC from phaeophorbide *a* requires the presence of a ferredoxin-dependent reductase, a soluble stroma protein responsible for the reduction of a double bond in the pyrrole system following the action of the ring-opening oxygenase. The transient intermediate in the PhaO reaction, a red bilin, is chemically identical to a terminal chlorophyll catabolite excreted by *Chlorella* (Gossauer, 1994; Hörtensteiner, Kräutler & Matile, unpublished; Fig. 6*a*).

3. *Gene expression in chlorophyll catabolism*

Experiments with inhibitors of protein synthesis indicate that the activation process unmasking chlorophyllase and Mg dechelatase during senescence requires new translation (Thomas *et al*., 1989), which in turn might mean that one or more important senescence-regulating genes are turned on. PhaO is not latent but its activation is sensitive to translation inhibitors, which suggests that it is made *de novo* in senescence (Schellenberg, Matile & Thomas, 1990). Mutants in three species (*Festuca pratensis*, *Phaseolus vulgaris*, *Pisum sativum*) display normal activation of chlorophyllase and dechelatase during senescence, but PhaO is not induced and leaf tissue remains green (Bachmann *et al*., 1994; Vicentini *et al*., 1995; Thomas *et al*., 1996). The coordinated action of several gene products might be necessary for invoking the chlorophyll catabolism pathway in senescence. One of these products (either encoded by the PhaO structural gene itself, or by a regulator of it) is missing or damaged in *stay-green* mutants, giving rise to the non-yellowing phenotype. In the case of *Festuca*, it was shown that LHC II complexes from plastids of the *stay-green* mutant comprise LHCP plus polar (phytol-less) chlorophyll derivatives (Thomas *et al*., 1989). Schoch & Brown (1986) observed that treating LHC II with chlorophyllase *in vitro* resulted in the formation of a stable chlorophyllid–protein complex. It is probable, therefore, that chlorophyll and its downstream catabolites remain complexed with protein right up until PhaO opens the macrocycle. At this stage the protein is liberated and becomes vulnerable to attack by proteases. Thus the principle of turnover-level regulation maintaining pigment–protein stoichiometry applies not only during complex formation but also when pigment–proteins are dismantled during gerontoplast development.

4. *Subcellular organization of chlorophyll catabolism*

A surprising recent finding is that much of the machinery of chlorophyll catabolism is located in the plastid envelope. Essentially all of the chlorophyllase and PhaO activity is associated with this membrane (Matile & Schellenberg, 1996), and the gerontoplast envelope also appears to be equipped with a carrier

that is responsible for exporting newly produced catabolites into the cytosol, since FCC is released into the medium if ATP is provided at the cytosolic face of the intact organelle (Matile *et al*., 1996). How does chlorophyll get from the thylakoid to the envelope? Are there specialized carrier proteins? Or do the pigment-binding proteins of thylakoid complexes become mobile? Might there be a role for the plastoglobuli (Tevini & Steinmüller, 1985) or similar bodies (Picher *et al*., 1993; Ghosh *et al*., 1994), providing hydrophobic, antioxidant-rich vehicles for moving chlorophyll between membranes? These questions cannot be answered yet, but the implications for the interlinking of the fates of pigments and proteins are clear.

5. *Interconversion of chlorophylls a and b*

A notable feature of the chemistry of the catabolites so far characterized is that they are all derivatives of chlorophyll *a* (Mühlecker & Kräutler, 1996). Phaeophorbide *b* is not a substrate for PhaO. And yet chlorophyll *b* is clearly broken down in senescence: how? Recently Japanese workers have described the conversion of chlorophyll *b* to *a* in developing plastids (Itoh *et al*., 1994; Itoh, Ohtsuka & Tanaka, 1996; Ohtsuka, Itoh & Tanaka, 1997). Schümann *et al*. (1996) also found that etioplasts could convert Zn-phaeophorbide *b* into Zn-phaeophytin *a*. Could this sequence or something similar be active in gerontoplasts? It might be relevant in this connection that the soybean *stay-green* variant *cytG* degrades chlorophyll *a* but chlorophyll *b* (and light-harvesting complexes) are relatively stable (Guiamét *et al*., 1991). Is *cytG* a mutation of a gene operating in the *b* to *a* pathway? If so, it offers a tempting target for molecular characterization of this step, since *cytG* is a cytoplasmic (presumably plastid DNA) gene.

Figure 4 presents the interconversion of *a* and *b* as a cycle, with implications not only for chlorophyll catabolism but also for steady-state adjustments, such as those occurring during light acclimation (see VII *3*.). It would not be surprising to find that the operation of the *b*–*a* cycle is accompanied by, and even the instigator of, dynamic changes in the proteins of thylakoid complexes.

X. CHLOROPHYLL AND ASSOCIATED PROTEINS IN SENESCENCE

1. *Thylakoid disassembly in gerontoplasts*

CO_2 assimilation decreases during leaf senescence, and it is well established that the limiting process determining photosynthetic rate over the period of decline is sited within the plastid and not, for example, at the level of stomatal resistance (Friedrich & Huffaker, 1980; Gay & Thomas, 1996). Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) is the single most important source of remobilizable protein N in the leaf and its degradation, accompanied by loss of CO_2 fixation activity, is a prominent feature of senescence (Makino, Mae & Ohira, 1984; Crafts-Brandner, Salvucci & Egli, 1990). The ultrastructure of senescing chloroplasts (that is, differentiating gerontoplasts) reveals a loss of stroma material as Rubisco disappears, but the most dramatic changes concern thylakoid membranes. Contact between the lamellae in grana stacks is loosened and the membranes disperse as the number and size of plastoglobuli increase (Tevini & Steinmüller, 1985). It is significant that although stroma and grana components are greatly altered, the plastid envelope retains integrity to the end of senescence (Butler & Simon, 1971; Thomas, 1977; Thomson & Whatley, 1980). Reorganization of thylakoid structure is accompanied by changes in photosynthetic light reactions and electron transport. The functions and protein complements of PS II and the cytochrome *b*/*f* complexes are particularly labile during senescence (Ben-David, Nelson & Gepstein, 1983; Holloway, Maclean & Scott, 1983; Woolhouse & Jenkins 1983; Roberts *et al*., 1987) but under normal circumstances components of different complexes are lost in a more-or-less coordinated fashion (Schmidt, 1988).

2. *Mobilization of chlorophyll–proteins in senescence*

I have argued that when proteins form complexes with their pigment chromophores they lock into stable structures. It follows that proteolytic remobilization during senescence requires the chlorophyll–protein complexes to be dissociated. Disassembly of pigment–protein complexes is potentially hazardous because it separates chlorophyll from the various mechanisms which, in the intact thylakoid, prevent photodynamic damage. We can now see how effectively metabolism via PhaO solves the problem. By opening the macrocycle and destroying the residual conjugated bond system in two virtually simultaneous reactions, phaeophorbide is rendered photodynamically impotent and the pigment-binding protein can be released for recycling. Even so, as in pigment–protein assembly, it is possible to invoke photodynamic damage during complex mobilization by excessive illumination or chemical interference (Thomas & Matile, 1987; Kar *et al*., 1993).

The interdependence of the degradation of chlorophyll and of the proteins that bind it is clearly exemplified in *stay-green* senescence mutants. Retention of pigment has been shown to be associated with immobilization of thylakoid proteins in non-yellowing variants of several species (Thomas & Smart, 1993; Bachmann *et al*., 1994; Guiamét & Giannibelli, 1994). This has important implications for the internal nitrogen economy of such plants.

Pigment–protein complexes in the thylakoids might account for over 30% of the total salvagable protein of chloroplasts. If chlorophyll degradation is impaired, much of the N of thylakoids is unavailable for recycling. It follows that, when N supply is limited, *stay-green* plants pay a significant penalty compared with normally yellowing types in terms of rates of growth and development, because their internal N relations are compromised by a large pool of inaccessible protein in senescent leaves (Bakken *et al*., 1996; Hauck, 1996). In this sense, it is not an exaggeration to consider chlorophyll catabolism as a pace-setting process with implications for whole-plant development and ecological fitness.

3. *Chlorophyll breakdown: catabolism or detoxification?*

Conversion of chlorophyll to FCC is a delicate operation upon which controlled redistribution of the N from pigment-binding proteins is absolutely dependent. The terminal products of chlorophyll breakdown are non-fluorescent tetrapyrroles (NCCs–Fig. 6*c*) which accumulate in the cell vacuole. The kinetics of FCC and NCC appearance, and a comparison of their molecular structures, suggest a precursor-product relationship (Matile & Kräutler, 1995; Matile *et al*., 1996). Radiolabelling has revealed that carbon from the pyrroles of chlorophyll is neither lost as CO_2 nor exported from senescent leaves to other parts of the plant but remains as terminal catabolites (Peisker *et al*., 1990; Matile *et al*., 1996). Moreover, Curty & Engel (1996) have recently shown that the total chlorophyll $a+b$ is converted mole-for-mole into a single *a*-type NCC in autumn leaves of *Cercidiphyllum japonicum* (further support for a *b* to *a* pathway). It may be concluded that plants catabolize chlorophyll into water-soluble porphyrin derivatives which accumulate in mesophyll cell vacuoles during foliar senescence. The N and C that get biosynthesized into the chlorophyll of a green cell are in that cell for good. In other words, the raw material of chlorophyll, unlike that of proteins and other constituents, is not salvaged during senescence for use elsewhere. It seems that this is the price to be paid for gaining access to the N of thylakoid proteins. Further features of the later stages of chlorophyll breakdown lead to an unusual conclusion.

Amongst the chemical structures of catabolites so far described are two conjugates. The NCC-2 of *Brassica napus* is a *ß*-glucoside and NCC-1 is malonated (Mühlecker & Kräutler, 1996–Fig. 6*c*). All NCCs are highly oxidized and hydroxylated. Recently the tonoplastic ATP-dependent transporter that delivers NCCs to the vacuole has been characterized (Hinder *et al*., 1996; Matile *et al*., 1996). Conjugation/hydroxylation and ATP-driven transfer to the vacuole are characteristic fates of xenobiotics in plant cells. Considering the conversions by which the intact macrocycle of chlorophyll is rendered photodynamically safe, and also the sequestration of breakdown products in the vacuole as terminal metabolites, it is difficult to escape the conclusion that this is a detoxification sequence rather than a catabolic pathway. The highly photodestructive properties of chlorophyll mean that it must be handled like a toxic compound by the cell in order to gain access to the considerable store of N invested in the chlorophyll-binding proteins.

XI. HOW DOES CHLOROPHYLL STABILIZE ASSOCIATED PROTEINS?

1. *Protein conformation*

It is clear that plant cells are equipped with the means to test apoproteins for the correctness of their associations with chlorophyll (or chlorophyllide, or protochlorophyllide, or phaeophorbide) and to destroy proteins that fail this test. What is it about such a chromophore that protects the complex from proteolysis? One possibility is that association between the pigment and its apoprotein moves hydrophobic zones, or refractory peptide bonds, to the outside of the complex, thus frustrating a waiting protease. Paulsen *et al*. (1993) found that folding of the light-harvesting protein is facilitated by chlorophyll. They overexpressed LHCP II in *E. coli* and reconstituted complexes with chlorophyll. Circular dichroism measurement indicated that the proportion of alpha helix increased from 20 to 60% when protein was renatured with chlorophyll. The renatured complex was resistant to attack by trypsin, in contrast to the protein alone. As well as wild-type LHCP, they overexpressed a form of the protein in which valine-229, in the 4th position from the C terminus (Fig. 2), was replaced with cysteine by site-directed mutagenesis. Where it is accessible to the reagent, cysteine in a polypetide chain is modified by the thiol label eosin maleimide. Paulsen *et al*. (1993) showed that renaturation with chlorophyll makes a cysteine residue in the membrane-spanning B helix inaccessible (which is not too surprising), but also the substitute cysteine at position 229. This residue is well beyond the D helix and hydropathy models would place it outside the hydrophobic core of the molecule, in a proteolytically-exposed position (Fig. 2). It seems that the stabilizing influence of the nearby chlorophyll (b3) extends to this part of the complex. The consequence of this for LHCP *in vivo* would be to limit proteolytic attack from the lumen direction. A possibly related observation here concerns the behaviour of cytochrome *f*.

2. *Cytochrome f*

Cytochrome*f* is significantly more stable in *stay-greens* than in yellowing genotypes (Davies *et al*.,

1990 ; Bachmann *et al*., 1994). The protein has a single membrane-spanning region, with most of the polypeptide chain exposed within the lumen. The decreased lability of cytochrome *f* is not explained by the thylakoid lumen's being a no-go area for proteolysis in *stay-green*s, because in *Festuca* the extrinisic, lumen-facing PS II protein OEC33 is degraded normally (Hilditch *et al*., 1989). We suggest that the luminal portion of cytochrome *f* is stabilized by its association with haem, just as the C terminus of LHCP II is made inaccessible by chlorophyll. Note the similarity between the structures of haem and chlorophyllide (Fig. 1). Does this mean that at least some of the haem is catabolized via the PhaO route and that disabling this pathway in *stay-green*s limits loss of this chromophore and degradation of the associated protein during senescence? It seems unlikely, but not impossible.

3. *Protease inhibition by tetrapyrroles*?

Facilitated folding into resistant conformations looks to be the probable mechanism by which chlorophyll and other tetrapyrroles confer stability on the proteins with which they are complexed, but there are other, additional, possibilities. One that needs further investigation is that tetrapyrroles are protease inhibitors. Vierstra & Sullivan (1988), investigating phytochrome turnover, found that haemin could inhibit proteolysis by the ubiquitin pathway. Binding haemin stabilizes part of the bovine serum albumin molecule against proteolysis, perhaps at least in part by directly limiting peptidolytic attack (Shin, Yamashita & Hirose, 1994). The hydrophilic macrocyclic derivatives of chlorophyll should be examined for similar regulatory effects on catabolic enzymes.

XII. CONCLUSION

It is estimated that more than 1% of the structural genes in the plant nuclear genome encode proteolytic enzymes and associated factors (Vierstra, 1996). The cytosol may be regarded as a highly lytic environment, in effect exerting severe selection pressure on proteins and multi-protein complexes to adopt resistant conformations in order to function stably over extended periods. The differential turnover of proteins and the modifying influences of substrates, effectors, prosthetic groups and ligands in general, are well described and perfectly consistent with the Darwinian view of survival on a molecular scale within the cell. It is also appreciated that many enzymes are much bigger than they need to be merely to carry out their catalytic function. The catalytically redundant portions of the polypeptide chain probably specify other important attributes such as subcellular localization, but particularly significant is likely to be the role they have evolved to play in surviving the proteolytic pressures of cytoplasmic existence. General rules relating protein structure and half-life have been proposed from time to time (see, for example, Ferreira & Davies, 1986; Chiang & Dice, 1988; Hersko & Ciechanover, 1992; Varshavsky, 1992), though, as in subcellular targetting, higher-order conformation is likely to be at least as decisive as amino acid composition or sequence. The exquisite poising of metabolism within the viable cell can be accounted for in part by the equilibrium between, on the one hand, stable protein conformation (for example, adequate substrate supply ensuring continuous occupation of an enzyme's active site, locking the entire protein into a refractory configuration) and, on the other, a constantly probing and testing proteolytic milieu. We see a clear demonstration of such a regulatory system in the post-translational turnover behaviour of POR in light and dark. I have argued throughout this review that, through their influence on protein conformation, chlorophyll and its derivatives play crucial, and often pace-setting, roles in the regulation of gene expression in the broad sense. The proposition could be extended to any and all metabolites that dock shape-specifically with proteins and multiprotein complexes. If a protein is gainfully employed in a structural or catalytic task, it is relatively safe. If not, it will be culled. What better way of attuning gene expression to the needs of metabolism and physiology?

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