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CHLOROPHYLL METABOLISM: FROM OUTER SPACE DOWN TO THE MOLECULAR LEVEL

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Key Word Index—chlorophyll synthesis; chlorophyll degradation; 5-aminolevulinate; protoporphyrin; protochlorophyllide.

Abstract—Recent developments in chlorophyll biosynthesis are reviewed and some attention is given to its further metabolism. © 1997 Elsevier Science Ltd

INTRODUCTION

Chlorophylls as essential pigments of photosynthesis have received much attention for a long time. Well-known highlights in the research of chlorophyll chemistry were the elucidation of its macrocyclic structure [1] and the chemical synthesis of chlorophyll *a* [2]. Less attention has been given the elucidation of the structure of phytol [3–5], in spite of the fact that this alcohol accounts for about 1/3 of the mass of chlorophyll.

Several reviews on a variety of aspects of chlorophyll biosynthesis have appeared within the last years [6–17]. The present review concentrates on recent progress in this field and emphasises several aspects that have not been treated in detail before. This is embedded in a short description of all the milestones along the biosynthesis pathway so that the non-specialist reader obtains an overview over the entire pathway of chlorophyll metabolism.

REMOTE SENSING

Chlorophyll biosynthesis is a mass process. Estimates for synthesis (and degradation) range up to 10^9 tons chlorophyll per year on our globe [18], about one third of this on land and two thirds in the oceans. Chlorophyll metabolism is the only biochemical process on our globe that can be observed from outer space. The principle is the same as for men's sensing pigments in our environment, namely differential reflection of light of different wavelengths. The observation of chlorophyll biosynthesis and degradation from space is especially useful for ecological research in the oceans but has also been applied to gross ecosystem production on land [19]. Changes in chlorophylls (and hence in phytoplankton) can be determined at any given time point within large areas. A practical application is detection by satellite of boundaries, or fronts, between phytoplankton-rich coastal water and clear ocean water. There fronts can rapidly change their positions. Since the boundary regions are rich in certain fish, e.g. tuna, fishermen are interested in their exact position at a given time. Aeroplanes or satellites designed for chlorophyll detection are usually equipped with several channels. An example: NIMBUS7, active from 1978 to 1982, was equipped with the coastal Zone Colour-Scanner (CZCS) containing five channels [20]. The reflection of light from chlorophyll-containing organisms is highest at the absorption minimum around 550 nm and lowest at the absorption maximum (Soret band) around 440 nm. Absorption at 440 nm includes also carotenoids. Two detector channels of the satellite are set at these wavelengths. The chlorophyll content is then calculated, after calibration and correction (see below), from the ratio reflection at 440 nm:reflection at 550 nm. The calibration requires the determination of the chlorophyll content in a given area (or rather volume!) by conventional laboratory methods. It should be recalled here that light is reflected only from the upper layer of the ocean, which is also the layer in which photosynthesis occurs. Thus no determination of chlorophyll is possible if photosynthetic organisms are transported into deeper water layers. A complication of the calibration is the fact that many marine organisms contain accessory photosynthetic pigments in addition to chlorophyll. The algorithm for the chlorophyll content has to be adapted in these cases. Other channels detect scattered light, basic reflection outside

It cannot be excluded at present, however, that a second pathway becomes operative only when the C_5 pathway of the plastids is blocked.

The C_5 pathway has been also found as the only pathway in archebacteria, cyanobacteria and most eubacteria. Only members of the alpha group of phototrophic bacteria, e.g. *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, and some non-phototrophic, Gram-positive bacteria synthesis ALA *via* the C_4 pathway [12, 38]. The C_4 pathway is the only pathway for ALA synthesis in animals and fungi. There it is localized in the mitochondria. Both pathways have been reported to operate in *Euglena gracilis* [39], in *Scenedesmus obliquus* [40], and in the soil bacterium *Arthrobacter globiformis* [41]. The gene encoding ALA synthase of the C_4 pathway has been named hemA gene. The same name, hemA gene, was also give to the gene encoding glutamyl-tRNA^{Glu} reductase of the C_5 pathway. The reason for this confusing nomenclature has been discussed [12].

Steps from ALA to protoporphyrin

Most of the subsequent steps of tetrapyrrole synthesis are identical in plants, animals and bacteria. Results obtained with one organism can therefore be considered as general. The steps include formation of the monopyrrole porphobilinogen from two molecules of ALA, formation of the linear tetrapyrrole hydroxymethylbilane from four molecules of porphobilinogen, ring closure, and two repeatedly, performed modification reactions of side chains. The steps are summarized in Fig. 2. Details of the reactions have been reviewed [17, 42, 43]. Only some properties are discussed here.

Competitive inhibition of ALA dehydratase by levulinic acid (LA) prevents metabolisation of ALA to tetrapyrroles and thus leads to measurable accumulation of ALA. The procedure has often been used for the investigation of ALA synthesis and its regulation. Instead of LA, several derivatives substituted at C-5 can be used (Fig. 3). Changes in the "upper part" of the molecule are not tolerated, however, for binding to the enzyme. Thus levulinic acid methyl ester, compounds with an extra methyl group or with substitution of one methylene group by the NH group (Fig. 3), do not inhibit the enzyme from *Rhodobacter sphaeroides* (W. Rüdiger, unpublished results). ALA dehydratase from *E. coli* was crystallized and the three-dimensional structure of the enzyme was elucidated [44]. The corresponding gene (hemB) was also identified in a plant (soybean), its cDNA sequence was determined [45].

Hydroxymethylbilane synthase (synonym: porphobilinogen deaminase) catalyses the condensation of four molecules of porphobilinogen to hydroxymethylbilane, a linear tetrapyrrole. The active enzyme contains a dipyrromethane cofactor that remains covalently bound to a cysteine residue during the enzymatic cycle [42]. Four additional porphobilinogen

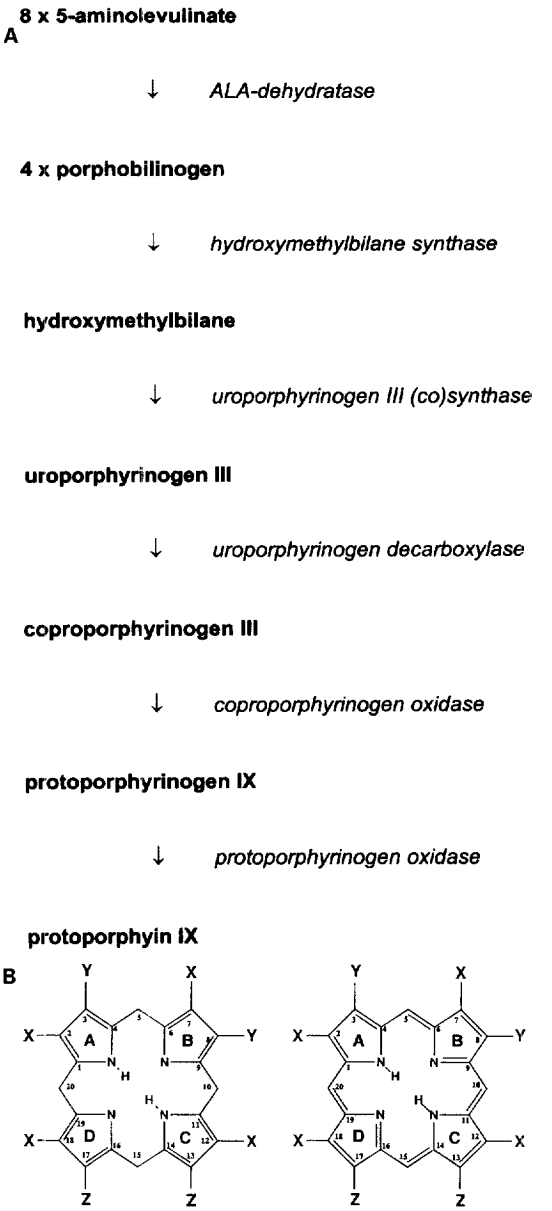

Fig. 2. Pathway from 5-aminolevulinate to tetrapyrroles. A. Intermediates and enzymes. B. Chemical structures of porphyrinogens (left) and porphyrins (right). Uroporphyrin(ogen) III: X = CH_2CO_2H, Y = Z = $CH_2CH_2CO_2H$. Coproporphyrin(ogen) III: X = CH_3, Y = Z = $CH_2CH_2CO_2H$. Protoporphyrin(ogen) IX: X = CH_3, Y = $CH{=}CH_2$, Z = $CH_2CH_2CO_2H$.

molecules are then linked to the cofactor until a hexapyrrole intermediate is formed. Release of the tetrapyrrole leaves the enzyme with the dipyrromethane cofactor for the next catalytic cycle. Hydroxymethylbilane synthase is the first enzyme of tetrapyrrole biosynthesis of which the crystal structure has been elucidated [46]. It is a flexible multidomain protein, conformation changes during the stepwise condensation of porphobilinogen have been detected [47]. Besides the cysteine residue that binds the cofactor, arginine and aspartate residues participate in the

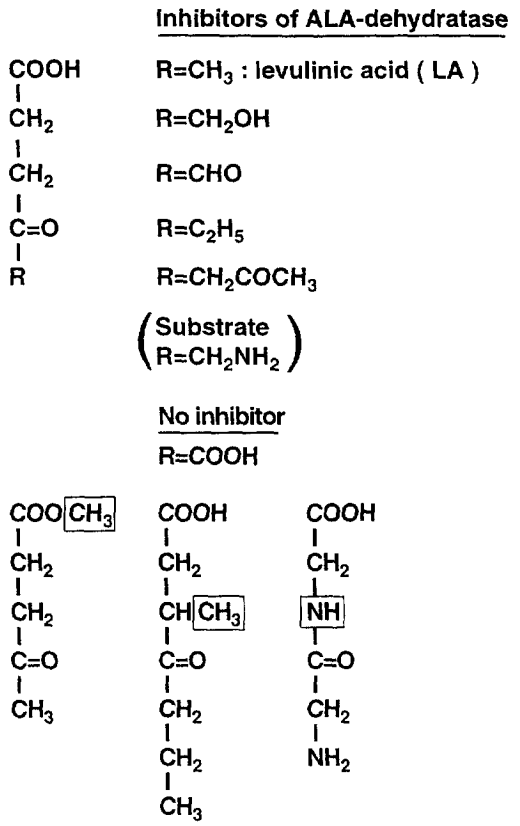

Fig. 3. Analogues of 5-aminolevulinic acid. Upper part: Inhibitors of ALA-dehydratase are (from top) levulinic acid, 5-hydroxylevulinic acid, 4,5-dioxovaleric acid, 4-oxohexanoic acid, 4,6-dioxoheptanoic acid. Lower part: Structures lacking inhibition of ALA-dehydratase include glutaric acid and (from left) levulinic acid methyl ester, 3-methyl-4-oxoheptanoic acid, glycylglycine.

catalytic reaction [48, 49]. The enzyme activity is inhibited by porphyrins [50] and by 5-hydroxymethylfurfural [51]. It remains to be shown whether such inhibition plays any role in the regulation of tetrapyrrole synthesis. The enzyme is confined to the plastids of higher plants according to immunochemical studies with antibodies raised against the overexpressed gene product from *Arabidopsis thaliana* [52].

The first cyclic tetrapyrrole in the pathway, uroporphyrinogen III, is formed from hydroxymethylbilane by uroporphyrinogen III synthase. Besides cyclisation, the enzyme catalyses inversion of one pyrrole ring *via* a spirocyclic intermediate [53]. The inverted ring becomes ring D in the porphyrin nomenclature [Fig. 2(B)]. The substrate hydroxymethylbilane is very unstable and easily undergoes spontaneous cyclisation to uroporphyrinogen I. This non-enzymatic reaction does not imply ring inversion, and uroporphyrinogen I is no substrate for uroporphyrinogen III synthase. These properties can explain the early observation that uroporphyrinogen III synthase, often named 'cosynthase' has to act in the presence of the previous enzyme in the pathway, hydroxymethylbilane synthase. All natural tetrapyrroles with a known biological function are derived from uroporphyrinogen III. Formation of uroporphyrinogen I (or of compounds derived from it) indicates malfunction (e.g. by mutation) of uroporphyrinogen III synthase or overflow of precursors e.g. after application of ALA [54]. It is interesting to note that acid-catalysed scrambling of uroporphyrinogens leads to al four possible isomers with the III isomer as the main product in the equilibrium [55, 56]. Thus the enzymes could have evolved in nature for specific production of that compound that was the main isomer in prebiotic times.

The next reactions are the stepwise decarboxylation of the four acetate side chains of uroporphyrinogen III to coproporphyrinogen III, catalysed by uroporphyrinogen decarboxylase, and the subsequent oxidative decarboxylation of two proprionate side chains at C-3 and C-8 yielding protoporphyrinogen IX (Fig. 2). The enzymes for these steps in high plants and their genes have recently been characterised.

A cDNA for uroporphyrinogen decarboxylase from tobacco was isolated and sequenced together with a partialclone from barley [57]. Southern blot analysis indicated a single copy gene in tobacco. The enzyme is synthesised with a presequence that is cleaved after import into the stroma of chloroplasts. Considerable amounts of the enzyme are present already in etiolated seedlings. Illumination for 6–8 hr resulted in the increase of the level of the enzyme. The enzyme activity decreases in the presence of CsCl at concentration >10 mM [58]. Other enzymes of the tetrapyrrole pathway are not affected. Since preincubation of plants with CsCl leads to more severe inhibition than incubation of the isolated enzyme, the authors proposed photooxidative damage of this and other enzymes mediated by the accumulated uroporphyrinogen after oxidation to uroporphyrin. This proposal was confirmed by constitutive overexpression of the full-length cDNA in reverse orientation in tobacco [59]. The anti-sense plants exhibited strong necrotic leaf lesions, correlated with the accumulation of uroporphyrin.

In a similar manner, full length cDNAs for the next enzyme in the pathway, coproporphyrinogen oxidase, were obtained from barley and tobacco [60]. The enzyme from tobacco was synthesised as 44.9 kDa precursor peptide. It was then imported into chloroplasts and processed to the mature size of 39 kDa. This import experiment confirmed the previous finding that the enzyme of higher plants is exclusively located in plastids [61]. Transformed tobacco plants that contained a full-length cDNA encoding coproporphyrinogen oxidase in inverse orientation, accumulated coproporphyrin (as a product of autoxidation of coproporphyrinogen) as expected and exhibited severe photo-oxidative damage [62].

The next enzyme in the pathway, protoporphyrinogen oxidase, is remarkable in two respects. Firstly, it is the first enzyme of plant tetrapyrrole biosynthesis that occurs in two different organelles,

plastids and mitochondria [63, 64]. Since location of all previous enzymes in the pathway is restricted to the plastids, the substrate protoporphyrinogen must be transported from the plastids to the mitochondria. Secondly, the substrate protoporphyrinogen is a colourless compound, as are all previous intermediates, and the product of the enzyme reaction, protoporphyrin, is the first pigment in the pathway. Porphyrin pigments are potential photosensitizers. Special mechanisms have envolved to protect plants from photodynamic damage mediated by these pigments [16]. The damage can be observed when such mechanisms are abolished.

A dramatic example is inhibition of protoporphyrinogen oxidase by diphenyl ether herbicides [65] leading to an uncontrolled formation of protoporphyrinogen IX. The accumulated protoporphyrinogen seems to diffuse throughout the plant cell. Like all porphyrinogens, it undergoes spontaneous autoxidation. The product of autoxidation, protoporphyrin IX, is lipophilic and accumulates in the membranes of the cell. This leads to destruction of tonoplast and plasmalemma in the light. The consequence is the complete loss of turgor within 1–2 hr in the light. Similar, but not as dramatic effects can be observed when protoporphyrin is accumulated after feeding plants with the tetrapyrrole precursor ALA and iron-complexing agents [66] albeit additional effectors besides protoporphyrin cannot be excluded in this case [67]. Leaf necroses were also detected after accumulation of uroporphyrin [58, 59] and coproporphyrin [62], as mentioned above. The damage is quite different from the photodamage that can be observed under carotenoid deficiencies [68, 69]. Such deficiencies can be caused by mutation or by inhibitors or carotenoid biosynthesis, e.g. by norflurazon. The direct photodamage is restricted to plastids in this case; chlorophylls are believed to act as photosensitizers. Other cellular compartments (cytoplasm, vacuole, mitochondria) seem unaffected. Impairment of expression of particular nuclear genes [68, 69] can be considered as a pleiotropic effect.

The protoporphyrinogen oxidase genes from yeast [70], *Myxococcus xanthus* [71] and *Arabidopsis thaliana* [72] have been cloned and characterised. Southern analysis indicated the occurrence of only one gene in *A. thaliana*. A leader peptide for import into mitochondria was deduced from the cDNA sequence. It remains to be shown whether there exist nevertheless two genes encoding the plant enzymes that occur in plastids and mitochondria [63, 64].

Insertion of magnesium

A landmark in the investigation of chlorophyll and bacteriochlorophyll biosynthesis was the detection and sequence analysis of a 46 kbp gene cluster, called 'photosynthesis gene cluster' from *Rhodobacter capsulatus*. The sequence was available in the EMBL data band since 1991 (accession number Z11165) and was published 4 years later [73]. This gene cluster contained *inter alia* the genes coding for all known enzymes of the Mg branch of the tetrapyrrole pathway. The nature of the enzymes was elucidated in several laboratories by defined gene disruption on the one hand and by functional analysis after overexpression in heterologous systems on the other hand [74]. Several of the homologous genes in cyanobacteria, algae or high plants were subsequently identified based on their sequence similarity.

Progress on this basis has been achieved with magnesium chelatase. Three genes (bchH, bchD and bchI) were found for three subunits of the bacterial enzyme [75, 76]. The homologous genes chlD, chlH and chlJ were identified in cyanobacteria [77]. In barley, Xan-f, Xan-g and Xan-h [78] are the homologous genes. Overexpression of the subunits in *E. coli* resulted in the active enzyme when all three subunits were combined [75, 77]. All subunits turned out to be soluble proteins. Previous reports of membrane localization of at least one subunit [79, 80] may have been caused by formation of a high-molecular complex that precipitated by centrifugation [75]. A re-examination of the localization revealed magnesium chelatase as a soluble stroma protein [81]. Mg chelatase requires ATP for its activity. It is probably needed for activation in connection with subunits BchI that contains an ATP binding motif [82] and BchD, the role of BchH is stoichiometric 1:1 binding of the substrate protoporphyrin [76]. The enzyme activity can be inhibited by mercurials (e.g. *p*-chloromercuribenzene sulphonate [83]), by hydroxybenzoic acid methyl esters [84] and by pheophorbide [85]. Since pheophorbide is a product of chlorophyll degradation (see below), inhibition of chlorophyll synthesis at the step of magnesium chelation could be physiologically significant in senescent plants.

Mg chelatase seems to be a 'bottle-neck' enzyme in the biosynthetic pathway of chlorophyll. In white tissues of variegated leaves of *Euonymus japonicus*, chelatase activity is probably blocked whereas enzymes of ALA, porphobilinogen and protoporphyrin formation are highly active [86]. In a number of mutants of three monocotyledonous and five dicotyledonous plants, limited chlorophyll synthesis resulting in lack of chlorophyll *b* formation was traced back with high probability to a block in magnesium chelatase activity [87]. The phenotype of such leaky mutants in chlorophyll synthesis strongly depends on environmental conditions, e.g. light intensity [88].

Mg chelatase is highly specific for Mg and excludes other metal ions, e.g. zinc, that is easily inserted nonenzymatically into porphyrins [89]. This is remarkable because later enzymes of chlorophyll synthesis accept zinc complexes of biosynthetic intermediates like the Mg complexes (see below). The significance of this metal ion specificity is not clear, especially since zinc-containing bacteriochlorophyll *a* was found as the main photosynthetic pigment in the acidophilic bac-

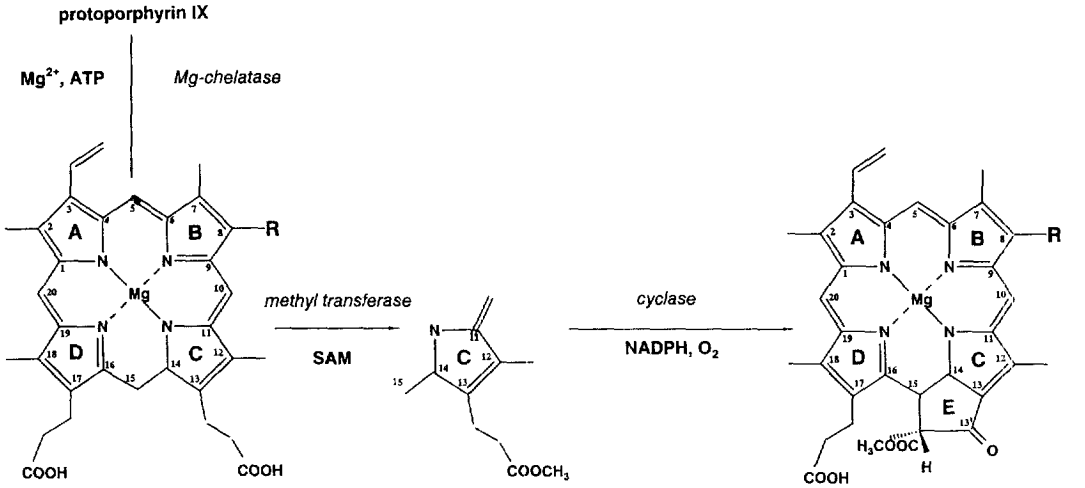

Fig. 4. Pathway from protoporphyrin IX to protochlorophyllide. SAM = *S*-adenosylmethionine.

terium *Acidiphilium rubrum* [90]. It should be remembered that the central Mg is easily removed from chlorophyll derivatives by acid but the central Zn is not. This might be the reason for metal exchange in an acidophilic organism.

Steps from magnesium protoporphyrin to protochlorophyllide

The step in the pathway after Mg insertion is formation of the monomethyl ester at the C-13 propionate side chain by *S*-adenosyl-L-methionine: Mg-protoporphyrin IX methyltransferase (Fig. 4). The reaction mechanism of the enzyme was investigated in detail (summarized by Richards *et al.* [91]), a 'ping-pong mechanism' was confirmed for the enzyme from wheat [92], but an 'ordered mechanism' for the enzyme from *Rhodobacter sphaeroides*. The bacterial enzyme is encoded by the bchM gene [93, 94]. Methyltransferase activity was demonstrated after heterologous expression in *E. coli*. The results led to a correction of the original assignment of the bchM gene to the bchM gene to the oxidative cyclase [95]. By complementation of a *Rhodobacter capsulatus* mutant defective in methyltransferase activity, the homologous chlM gene from the cyanobacterium *Synechocystis* PCC 6803 was detected [96]. The ChlM protein product exhibits 29% sequence identity to BchM. The mechanism of the cyanobacterial enzyme has still to be investigated. In a coupled assay using chloroplasts from *Chlamydomonas rheinhardtii*, addition of *E. coli* extract containing heterologously expressed recombinant methyltransferase increased formation of the monomethyl ester from the substrate Mg protoporphyrin but did not increase formation of protochlorophyllide [97]. This indicates that the methyltransferase reaction is normally not a rate-limiting step in the reaction chain of tetrapyrrole biosynthesis. The nucleoside analogue sinefungin was found to inhibit the methyl transferase in barley leaves [98]. The result was a strong inhibition of chlorophyll biosynthesis.

The enzyme activity responsible for formation of the isocyclic ring of chlorophylls (Fig. 4) has been investigated repeatedly since 1968 (reviewed by Porra [17]). The plant enzyme requires NADPH and molecular dioxygen. Incorporation of $^{18}O_2$ into the 13^1-carbonyl group has been demonstrated in plants [99]. Two pathways have been found in bacteria. In anaerobic bacteria, oxygen at C-13^1 is derived from water [100] but in aerobic bacteria it is derived from dioxygen [101]. The enzyme activity, studied *in vitro* with isolated plastids of *Chlamydomonas rheinhardtii*, was not inhibited by CO, KCN or NaN_3 [97, 102]. However, iron chelators inhibited both the enzyme from *C. rheinhardtii* and from *Synechocystis* PC 6803 indicating that non-heme iron could be involved. Two protein compounds, a soluble and a membrane faction, were described for the cyanobacterial enzyme [102]. So far, no gene has been detected for any subunit of the aerobic cyclase enzyme. In the gene cluster of *Rhodobacter capsulatus*, the bchE gene has been assigned to a subunit of the anaerobic cyclase [93].

The bchJ gene of *Rhodobacter capsulatus* was demonstrated to encode a vinyl reductase [103]. The experimental evidence for this conclusion was a shift in protochlorophyllide pools from the monovinyl to the divinyl compounds in null mutants of the bchJ gene. Less bacteriochlorophyll was formed in the mutant indicating that monovinyl protochloropyllide is a better substrate than the divinyl compound for the next step in *R. capsulatus*, viz. the dark reduction of ring D. The data indicated that the product of the bchJ gene codes for a protein that is involved in reduction of the 8-vinyl group of protochlorophyllide. The results implicated furthermore another (or even more) vinyl reductase(s) encoded by different gene(s) that were not knocked out in the null mutant. The authors speculated that other vinyl reductase(s) could have different substrate specificity so that reduction of the vinyl

group catalysed by the hitherto unknown enzyme(s) occur with substrates located before or after protochlorophyllide in the tetrapyrrole pathway.

Since several chlorophyll precursors have been found in algae and higher plants as divinyl and as monovinyl, monoethyl derivatives, parallel pathways for chlorophyll biosynthesis have been proposed [104]. The significance of obligatory vinyl reductases can be questioned. A marine photosynthetic bacterium related to *Prochloron*, contains [8-vinyl]chlorophyll *a* [105], and a maize mutant that accumulates considerable amounts of [8-vinyl]chlorophylls *a* and *b* is photosynthetically active and viable [106, 107].

Formation of the chlorin macrocycle

Two pathways can clearly be distinguished physiologically for the next step, the hydrogenation of ring D leading from protochlorophyllide to chlorophyllide [Fig. 5 (A)]. Knowledge about enzymes and genes for this step has recently been reviewed [108]. In one pathway this step occurs in the dark, and in the other pathway it requires light. The dark pathway is considered to be the phylogenetically older one. It is the only pathway found in phototrophic bacteria but it occurs (together with the light-independent pathway) in algae, mosses, ferns and gymnosperms. The light-dependent pathway seems to be ubiquitous in organisms performing oxygenic photosynthesis, from cyanobacteria [109, 110] to angiosperms.

The products of three genes (bchB, bchL and bchN) are needed for protochlorophyllide hydrogenation in the dark by phototrophic bacteria. Analogous genes with 35–53% sequence identity have been detected in organisms up to gymnosperms [74, 111, 112]. The probability to mutate one of these three genes in green algae must be very high; such mutants that have often been obtained e.g. of *Chlamydomonas*, *Chlorella* and *Scenedesmus* [113], lack the ability to accumulate chlorophyll in the dark but turn green in the light. The same effect had a deletion of the chlL gene in the cyanobacterium *Synechocystis* PCC 6803 [114]. The role of the single subunits in the hydrogenation reaction is not yet clear. *Vice versa*, the *Chlamydomonas* pc-1 mutant which is incapable of protochlorophyllide conversion in the light, has a frameshift mutation in the gene encoding the light-dependent reductase [115]. The POR enzyme (see next section) is not detectable in the mutant.

The light-dependent reaction has mainly been investigated in angiosperms [113, 116]. The enzyme NADPH:protochlorophyllide oxidoreductase (POR), the main protein in prolamellar bodies of etioplasts, forms a ternary complex with its substrates, protochlorophyllide and NADPH. The enzyme-bound pigment can be considered as the photoreceptor triggering its own hydrogenation reaction. At least one photon per protochlorophyllide molecule is required for the reaction, i.e. the reaction proceeds with protochlorophyllide in the excited state or a photoproduct derived thereof, and not with the pigment in the ground state [117]. In a scheme originally developed by *in vitro* studies [118] (reviewed by Rüdiger and Schoch, [6]; Schulz and Senger, [113]), illumination of the ternary complex induces hydrogen transfer from the pro-S face of NADPH to C-17 of protochlorophyllide [119] followed by a proton transfer to C-18 from water or tyrosin of the enzyme [120] forming $NADP^+$ and chlorophyllide. The oxidised cofactor is then replaced by NADPH followed by release of chlorophyllide and binding of new protochlorophyllide. Each step is detectable by a defined spectral shift. Protochlorophyllide in the ternary complex exhibits 2 absorption maxima at about 638 and 652 nm and 77 K fluorescence emission maxima at about 642 and 657 nm, respectively. The two spectral forms are supposed to be monomers (638/642 nm) and aggregates (652/657 nm) of the ternary complex [121, 122]. The aggregates are preserved by ATP and by the phosphatase inhibitor fluoride indicating involvement of protein phosphorylation in the aggregation [123]. Protochlorophyllide absorbing at about 630 nm (77 K fluorescence emission at 633–636 nm) is believed to be free pigment or pigment not bound to the active centre of the enzyme, it is not directly phototransformable. Such 'short-wavelength' protochlorophyllide was found to occur naturally in hypocotyl and roots of bean seedlings [124] and in pea epicotyls [125].

Circular dichroism was used to study the aggregation state [121, 122], secondary structure [126] and the substrate specificity [127] of the enzyme. The absolute configuration of natural protochlorophyllide *a* was recently determined to be $13^2(R)$ [127]. This is the same configuration that is also found in chlorophyll *a*. The unnatural enantiomer $13^2(S)$ protochlorophyllide *a* (protochlorophyllide *a'*) was prepared chemically. It turned out to be no substrate for the POR preparation consisting of the solubilized fraction of prolamellar bodies from wheat etioplasts. The central Mg could be substituted by Zn without loss of acceptance by POR. However, any substitution at $C\text{-}13^2$ resulted complete loss of substrate property [127]. This unexpected result suggests that the isocyclic ring might somehow be involved in the photoreduction of ring D, possibly *via* an enolate-type intermediate [117]. On the other hand, modification at other sites of the molecule, e.g. formyl instead of methyl at ring B, was tolerated by POR indicating that the branching point for chlorophyll *a* and chlorophyll *b* synthesis could be located before photoreduction of ring D [128].

It has been known for several years that illumination of etiolated barley seedlings resulted in degradation of most of the POR protein and disappearance of nearly all POR mRNA (reviewed by Schulz and Senger [113]). However, in cucumber seedlings only a transient decrease in POR mRNA, POR protein and activity level was detected upon transition from dark

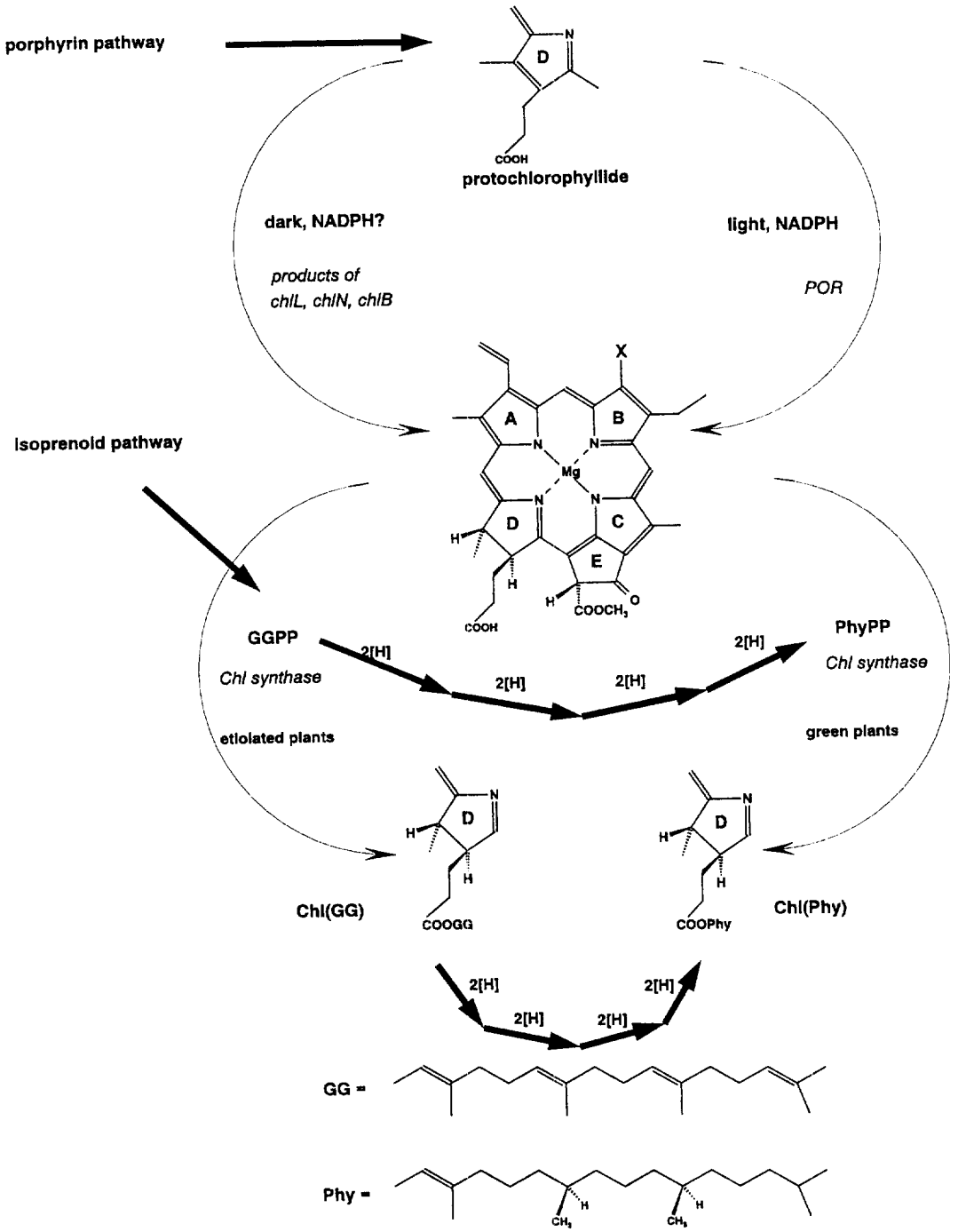

Fig. 5. A. Two pathways exist for reduction of protochlorophyllide and for esterification of chlorophyllide. Chlorophyllide *a*: X = CH_3; chlorophyllide *b*: X = CHO.

to light. After 1–2 hr of continuous illumination, a strong increase in POR mRNA, protein and activity levels was observed culminating 12 hr after the beginning of illumination [129]. Other authors found only a light-dependent increase of POR mRNA in cucumber seedlings [130]. Treatment with benzyladenine or gibberellin A_3 resulted in the increase, treatment with abscisic acid in the decrease of POR mRNA levels in the dark. The seeming discrepancy between previous and recent result can possible be explained by the finding of two light-dependent enzymes, POR A and POR B, that have been characterised in barley and *Arabidopsis* seedlings [131–133]. Transition from darkness to light conditions leads to a decrease in the mRNA level of POR A but not of POR B. In green barley plants grown in day/night cycles, POR A

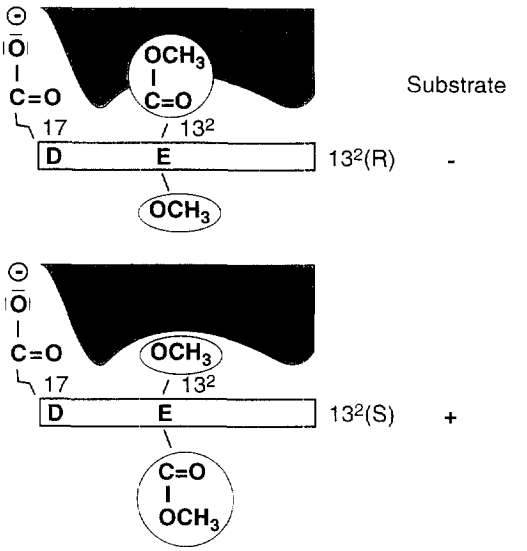

Fig 5. B. Substrate specificity of chlorophyll synthase. Large substituents at the 'upper side' are not tolerated by the enzyme.

mRNA and protein levels increase towards the end of the night phase. In a 'de-etiolated' (det) mutant of *Arabidopsis*, the level of POR A mRNA and protein is constitutively down-regulated and no long-wavelength form of protochlorophyllide is formed whereas POR B is normal [134]. The mutant is very sensitive to photooxidation when illuminated but slowly turns green in dim light. POR A can also be down-regulated in continuous far-red light mediated by phytochrome A [135]. This down-regulation does not occur in a phytochrome A-deficient mutant.

Further modifications of side chains

Biosynthesis of chlorophyll *a* is completed by esterification of chlorophyllide *a* (see next section). Biosynthesis of chlorophyll *b* requires an additional step, the transformation of the methyl group of ring B into a formyl group. The formyl oxygen is derived from molecular oxygen in the green alga *Chlorella* [136] and in maize [137]. Detection of non-esterified precursors of chlorophyll *b* [138–140] and the study of esterification of chlorophylls *a* and *b* in green tobacco cell cultures [141] point to incorporation of oxygen before esterification. One has to keep in mind, however, that the oxygenase enzyme has not yet been isolated and no *in vitro* studies on substrate specificity have been performed. The reverse reaction, reduction of the formyl group to the methyl group, has been studied, *in vivo* and *in vitro* [142–144]. It can be performed with the non-esterified chlorophyllide *b* and with the esterified chlorophyll *b* or their zinc analogues [145]. The reaction can easily be achieved by chemical reduction provided that a central metal (e.g. Zn) is present [146]. The significance of the transformation of chlorophyll *b* to *a* in plants is still under debate. The mutual transformation of chlorophyll *a* and *b* and *vice versa* ('chlorophyll cycle') could be significant for acclimation of plants to varying light conditions. The chlorophyll *b*- to *a*-transformation could be involved in chlorophyll degradation in senescing leaves (see below). The pathways for other modifications of side chains that must occur in biosynthesis e.g. of chlorophyll *c* and *d* are still unknown, but likely steps are discussed here. Chlorophyll *d* is the major pigment in a new oxygenic photosynthetic prokaryote [147]. It has a formyl group at C-3 instead of a vinyl group but otherwise is identical with chlorophyll *a*. Oxidation of the 3-vinyl group of chlorophyll *a* or a precursor of chlorophyll *a* is a likely reaction. The name 'chlorophyll *c*' is used for a group of pigments that are widely distributed in chromophyte algae. The name is misleading, compounds classified under this name are no chlorins but true porphyrins, and they are not esterified. A more adequate name is 'protochlorophyllide *c*'. The C_1-compound is the monovinyl and the C_2-compound is the divinyl compound, corresponding to monovinyl and divinyl protochlorophyllide *a*. The propionate side chain at C-17 of protochlorophyllide *a* is modified in the *c*-compounds into an acrylate side chain. Since the cyclase reaction with Mg protoporphyrin monomethyl ester that creates ring E is believed to proceed via modification of the propionate side chain at C-13 into an acrylate side chain [6], such a reaction can be postulated for the side chain at C-17, too.

Chlorophyllide *a* is an intermediate not only in the biosynthesis of chlorophylls of higher plants but also in the biosynthesis of bacteriochlorophylls. Chlorophyllide *a* can be further modified by either one of 2 enzymes. The product of the bchF gene catalyses the hydroxylation at the 3-vinyl group forming [3(2-hydroxyethyl)] chlorophyllide *a*. Alternatively, the products of the bchXYZ gene cluster catalyse hydrogenation or ring B forming [3-vinyl]-bacteriochlorophyllide *a* [94]. Due to the relaxed

specificity of these enzymes, hydrogenation of ring B can also take place with [3(2-hydroxyethyl)] chlorophyllide *a*. Alternatively, hydroxylation of the vinyl group can occur after hydrogenation of ring B. Dehydrogenation of the 2-hydroxyethyl group to the acetyl group at C-3, forming bacteriochlorophyllide *a*, is then catalysed by the gene product of bchC [94].

The prenylation step

The last step of chlorophyll biosynthesis before incorporation into protein complexes is the esterification [Fig. 5(A)]. This reaction increases the lipophilic character of the pigment and, as deduced from three-dimensional structures of chlorophyll protein complexes [148–154] leads to specific interactions with the peptide chains. Such interactions can also be expected in chlorophyll-protein complexes for which the resolution of the X-ray structure does not yet allow a clear statement [155]. The significance of the long-chain alcohol for pigment-protein interactions was confirmed e.g. by exchange of single pigments in bacterial reaction centres [156], reconstitution of chlorophyll *a*/*b*-binding proteins [157] and stabilisation of newly-formed chlorophyll *a*-binding proteins [158–160].

The esterification reaction, catalysed by chlorophyll synthase, requires substrates from two pathways, chlorophyllide (or bacteriochlorophyllide) from the porphyrin pathway and phytyldiphosphate (or geranylgeranyldiphosphate) from the isoprenoid pathway [Fig. 5(A)]. Enzyme activity has been detected in the inner membranes of etioplasts [161] and in the thylakoid membrane of chloroplasts [162]. Early work (summarized in [163–164]) had shown that illumination of etiolated plants results at first in formation of chlorophyll that is esterified with geranylgeraniol and that this pigment is apparently stepwise reduced, chlorophyll esterified with phytol being the end product. In green plants, newly-formed chlorophyll contains phytol from the beginning. This means that reduction of geranylgeraniol to phytol must have occurred before esterification in green plants, i.e. already with the diphosphate. Thus two parallel pathways seem to exist also for the last steps of chlorophyll biosynthesis [Fig. 5(A)]. The enzyme of etioplasts accepts chlorophyllide *a* and chlorophyllide *b* equally well but does not accept bacteriochlorophyllide. The central metal can be Mg of Zn, but Cu, Co or Ni are not tolerated nor is the metal-free pheophorbide a substrate. Application of several modified chlorophyllides revealed the importance of position 13^2 [165]. In chlorophyllides (and chlorophyll), substituents at C-13^2, C-17 and C-18 protrude from the tetrapyrrole plane. Bulky substituents at C-13^2 have to be on that side of the porphyrin plane, which is opposite to the side of the propionate side chain at C-17 [Fig. 5(B)]. The authors discuss the lack of binding substrates with bulky substituents on the 'wrong' side by steric hindrance. One consequence is the lack of esterification of chlorophyllide *a'*, the epimer of chlorophyllide *a* [165]. If chlorophyll *a'* is a natural compound in reaction centre I [166], it must then be formed after esterification by epimerization of chlorophyll *a*.

Disruption of the bchG gene of *Rhodobacter capsulatus* by interposon insertion resulted in a mutant strain that produced bacteriochlorophyllide instead of bacteriochlorophyll [94, 167]. This indicated that the bchG gene encodes bacteriochlorophyll synthase. Since the consequence of disruption of the bchP gene which is located directly downstream of bchG, was accumulation of bacteriochlorophyll esterified with geranylgeraniol [94], it was concluded that geranylgeranyl diphosphate was the preferred or the obligatory substrate for bacteriochlorophyll synthase. A reaction sequence similar to that in etiolated plants was postulated for *R. capsulatus*, such that reduction of geranylgeraniol to phytol should occur only after incorporation of the alcohol into the pigment. Heterologous expression of the bchG of *R. capsulatus* and the homologous chlG gene of *Synechocystis* PCC 6803 in *E. coli* allowed *in vitro* enzymatic assays with several substrates [168]. As expected, the chlG gene product accepts chlorophyllide but not bacteriochlorophyllide and, *vice versa*, the bchG gene product accepts bacteriochlorophyllide but no chlorophyllide as substrate. Furthermore, it was found that both the bchG and chlG gene products prefer phytyl diphosphate over geranylgeranyl diphosphate, analogous to the pathway in green plants and different from that in etiolated plants.

The first putative chlorophyll synthase gene of higher plants, G4, was found in *Arabidopsis thaliana* [169]. Heterologous expression of the G4 gene from *A. thaliana* in *E. coli* revealed the expected chlorophyll synthase activity and preference for geranylgeranyl diphosphate over phytyl diphosphate [170], similar to the enzyme specificity in etiolated plants. Since it was determined as single copy gene, being expressed in greening and green plants [169], it remains to be shown whether there exist different gene products in etiolated and green plants or whether the substrate specificity changes with the environment, e.g. the lipid composition of the membrane. Two different bchG homologues were detected in *Chloroflexus aurantiacus* [171, 172, Gen band accession number Z 34000]. Based on the clustering of the genes, it was speculated that one of these chlorophyll synthases is specific for bacteriochlorophyll *a* synthesis in the reaction centre and inner antennae and the other for bacteriochlorophyll *c* synthesis in the chlorosomes [171]. Alignment of the known chlorophyll synthase proteins revealed a relationship to polyprenyltransferases involved in ubiquinone and menaquinone biosynthesis [171].

CHLOROPHYLL DEGRADATION

Global chlorophyll degradation equals global chlorophyll synthesis, estimated to come to 10^9 tons

per year [18]. One must distinguish the chlorophyll breakdown after cell death from that occurring in living cells. The former process can happen e.g. during digestion of plant tissue in the intestine of herbivores, in leaves that naturally fall down or are somehow detached from plants while still green, or in dead marine organisms from macroalgae to phytoplankton. This chlorophyll breakdown probably does not follow one specific pathway in all these cases. It is generally a slow process so that modified tetrapyrroles can be found for a long time. The porphyrins found in fossil fuels are believed to be derived from such slow chlorophyll breakdown. In contrast, the loss of the green colour of leaves in autumn can be extremely fast. This is conceivable considering the photodynamic properties of porphyrin pigments including free (i.e. not protein-bound) chlorophyll. The fast degradation to colourless products enables the plants to recycle the nitrogen of the thylakoid proteins. The significance of chlorophyll degradation was stressed from a different point of view in a review by mentioning the well-known colours of autumn, that attract more than 50% of the annual number of visitors in certain tourist regions, with the words: 'Such is the power of chlorophyll destruction!' [18]. The authors who carefully compiled the literature on chlorophyll breakdown before 1987, stated that the pathway of degradation is a 'biological enigma'. Since then, considerable progress has been made in the knowledge of breakdown products and enzymes (reviewed [173–176]). Experimental systems to study chlorophyll breakdown implied green plants kept in darkness for several days, senescing cotyledons or leaves in the light, ripening fruits, or green algae transferred from autotrophic to heterotrophic growth conditions. Even if the particular products in the different experimental systems are not entirely identical, some basic steps that seem to be general can be extracted from the available data.

The first two steps are removal of phytol, catalysed by chlorophyllase, and removal of magnesium, catalysed by magnesium dechelatase. The order in which these two steps occur is not well established, and can vary from plant to plant [177]. In most cases studied so far, the chlorophyllase reaction precedes the dechelatase reaction, however.

Chlorophyllase is a well-characterised plastid enzyme [178–181]. The intraplastidal localization of chlorophyllase has not yet been elucidated. Most authors assumed that the enzyme is located in the thylakoid membrane. However, in density gradients and on 'green gels', chlorophyllase activity was detected very close to the light-harvesting complex of photosystem II but does not seem to be associated with this or any other protein complex of the thylakoids [182, 183]. Depending on the experimental conditions, chlorophyllase activity was also detected in other fractions including the plastid envelope. It is questionable whether the detectable enzyme activity belongs in all cases to the same enzyme protein. Maxima of chlorophyllase activity have repeatedly been found not only at stages of high chlorophyll degradation but also at stages of high chlorophyll synthesis [184]. The phytol residue is probably not stored as free alcohol but either esterified with acetic acid [185] or transformed into several oxygen-containing compounds that are probably products of photooxidation of phytol [186].

Magnesium dechelatase activity has been demonstrated in senescent leaves and in mature green cotyledons [187, 188]. Cytoplasm protein synthesis is required for maximum activity [187]. The dechelatase seems to be associated with complexes of photosystem I [182]. However, removal of Mg from chlorophyllide (but not from chlorophyll!) was also catalysed by a low-molecular weight compound, extracted from *Chenopodium album* [189]. Incubation conditions for optimum activity, including the pH optimum, were different from that for the enzymatic reaction [188]. It remains to be shown which role this low-molecular weight compound plays in chlorophyll metabolism, in relation to the enzyme activity described above. It remains an enigma how pheophytin that occurs in photosystem II is made. If Mg dechelatase only accepts non-esterified pigments [187, 188] and if also the low-molecular compound does not remove Mg from esterified chlorophyll [189], the pheophorbide is the only product of these biological reactions. However, pheophorbide is not a suitable substrate for chlorophyll synthase. Thus no pheophytin will be produced in this way. The easiness of non-enzymic Mg loss from chlorophyll *a* could indicate a non-enzymic pathway for pheophytin formation, but catalysis by another enzyme, not yet detected, is likely, too.

The key step of chlorophyll degradation is the oxidative opening of the chlorophyll macrocycle. This reaction and the structures of the resulting products are the subject of several recent reviews [173–176]. According to incorporation of ^{18}O into a product of chlorophyll degradation excreted by the green alga *Chlorella protothecoides*, this step is catalysed by a mono-oxygenase [190]. The reaction that was studied *in vitro* with senescent plastids (gerontoplasts) of higher plants, requires oxygen, reduced ferredoxin and stromal protein. It is specific for pheophorbide *a* and does not occur with pheophorbide *b* [191–193]. The enzyme is located in the envelope membrane of gerontoplasts [194]. The enzyme is considered as key enzyme for chlorophyll degradation, because its activity can be detected only after onset of senescence. Activity of chlorophyllase and Mg dechelatase are detectable already in pre-senescent green tissue. A non-yellowing mutant (synonym: stay-green mutant) of *Festuca pratensis* was demonstrated to be defective in pheophorbide oxygenase and not in the other enzymes of chlorophyll breakdown [195]. Under conditions of senescence, this mutant is unable to degrade the proteins of chlorophyll-protein complexes whereas all other proteins are normally degraded [173]. This result emphasises not only the role of pheophorbide

oxygenase as key enzyme of chlorophyll degradation, but also the role of chlorophylls for stabilisation of chlorophyll-protein complexes. The primary degradation products of pheophorbide oxygenation are colourless, fluorescent compounds that are transformed in a fast reaction into non-fluorescent, more stable catabolites. These catabolites are transported in an ATP-dependent manner into the vacuole [196]. They are not further degraded within the plant so that their concentration in a yellow leaf equals the concentration of chlorophylls in the same leaf before senescence.

Several structures of chlorophyll catabolites from algae [197–199] and higher plants [200–202] have been elucidated. A tentative reaction mechanism based on the structures has been proposed [174]. A common feature is the structure of a 19-formyl-1 [21H, 22H]-bilinone derivative (Fig. 6). This structure is the direct consequence of the oxygenase reaction. The monooxygenase mechanism, determined for the alga [190], is probably true for higher plants, too. The red products excreted by the alga into the culture medium [Fig. 6(A)] are probably more closely related to the primary product of the oxygenase reaction than the colourless products of higher plants [Fig. 6(B)]. Products derived from both chlorophylls *a* and *b*, have been detected among the algal products. The main modification seems to be the hydrolysis of the methyl ester group at ring E, probably catalysed by an esterase. The free carboxylic group is readily decarboxylated, as common for β-ketoacids. Further products, not mentioned in Fig. 6(A), can be derived from these products by isomerisation reactions [174]. The catabolites of higher plants [Fig. 6(B)] must have been hydrogenated at some metabolic step. Since catalytic hydrogenation and proton-catalysed rearrangement of the algal pigment results in products with the same carbon skeleton as that of chlorophyll catabolites from higher plants [176], a reductase reaction after ring-opening is proposed for higher plants [174]. Further modifications involve hydroxylation reactions, in *Brassica napus* only at the ethyl group of ring B, in *Hordeum vulgare* at side chains of ring A and B [Fig. 6(B)]. This modification probably favours transport into the vacuole and storage, in agreement with formation of the β-glucoside and the malonic acid ester in *B. napus*.

So far degradation of chlorophyll was discussed only down to the step of excretion by algal cells or of transport into the vacuoles of higher plants. The tetrapyrroles are not further metabolized within the plant, contrary to the leaf proteins that are recycled. Since the nitrogen content of chlorophyll is only about 3–5% of the nitrogen content of leaf proteins [203], lack of recycling of tetrapyrroles has no significant impact on the nitrogen balance of plants. For the global metabolism of chlorophyll, further degradation of the chlorophyll catoblites must be achieved by micro-organisms either in the soil or in aqueous environment. Reaction mechanisms or products of such reactions are still unknown but one can expect a variety of mechanisms rather than a uniform mechanism used by the variety of micro-organisms that further metabolize the known chlorophyll catabolites.

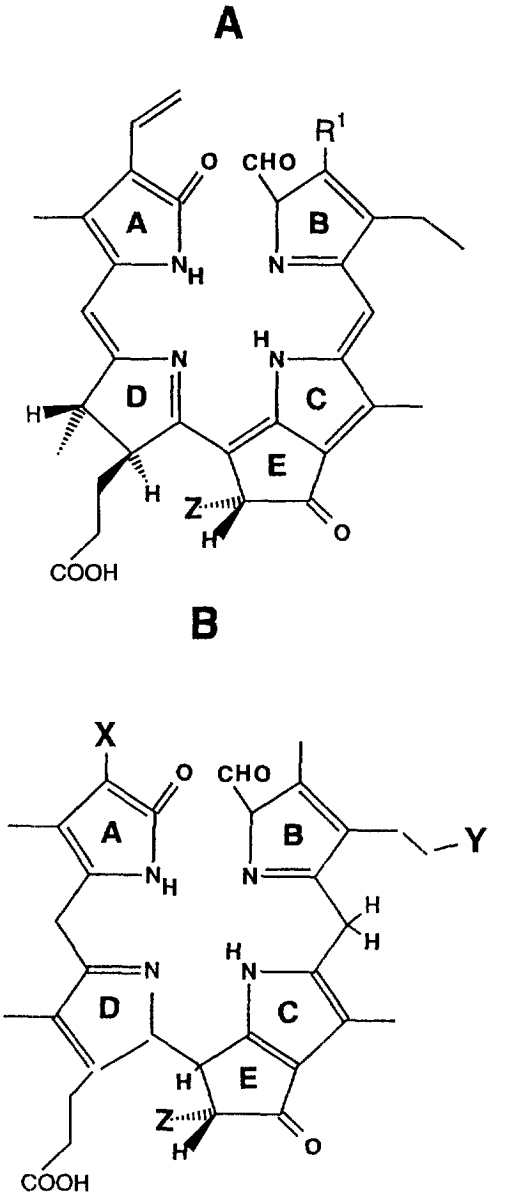

Fig. 6. Degradation products of chlorophyll. A. Products excreted by *Chlorella protothecoides*, $R^1 = CH_3$ or CHO; Z = H, CO_2H or CO_2CH_3. B. Products isolated from *Brassica napus* (X = $CH{=}CH_2$, Y = OH, *O*-β-glucose or $OCOCH_2CO_2H$, Z = CO_2H) and from *Hordeum vulgare* (X = $CHOHCH_2OH$, Y = OH, Z = CO_2CH_3).

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